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Is the ability to regenerate damaged skeletal muscle genetically determined? : Can allelic variation in the gene, PAX7, explain observed differences in recovery of force following damage by eccentric exercise?

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IS THE ABILITY TO REGENERATE DAMAGED SKELETAL MUSCLE GENETICALLY DETERMINDED?

CAN ALLELIC VARIATION IN THE GENE, *PAX7*, EXPLAIN OBSERVED DIFFERENCES IN RECOVERY OF FORCE FOLLOWING DAMAGE BY ECCENTRIC EXERCISE?

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ABSTRACT

High force eccentric muscle actions, during forcibly lengthening contracting muscle, have been found to produce pronounced muscle damage, especially if exercise is unaccustomed. A cascade of events is initiated in order to repair damaged skeletal muscle tissue including degeneration and regeneration processes. For the duration of muscle regeneration, satellite cells are the primary source of new myoblasts in adult muscle. This process requires activation of quiescent mononucleated satellite cells which proliferate, differentiate and fuse together to form fully functional muscle fibres. The satellite cells are specified by the gene, *Pax7*, during development. Experimental studies of *Pax7* null mice that lack the *Pax7* gene revealed that after birth there is little or no skeletal muscle growth or regeneration. Recently polymorphic repeats have been determined within the promoter and intronic regions of the human *PAX7* gene. The promoter polymorphism has been shown to alter the transcription efficiency of this promoter *in vitro*.

It is of great interest to note that efficiency of repair of muscle damage varies considerably between individuals. This leads to a conclusion that variation in *Pax7* polymorphic sequences between individuals could affect skeletal muscle regeneration by virtue of the crucial role of *Pax7* in satellite cell specification. We have measured allelic variation of *Pax7* (promoter and intronic) in population groups and in volunteers undergoing forced exercise induced damage. Preliminary data suggests that there is a significant variation in allele frequencies between the study population groups. With

respect to promoter polymorphisms, there is a trend toward high responders being associated with allele 8 (8 CCT repeats) and a trend towards low responders being associated with allele 11 (11CCT repeats). On the other hand, allelic variation with respect to the *PAX7* intronic polymorphism cannot explain the observed differences in recovery of force following damage by eccentric exercise.

ACKNOWLEDGMENTS

My honours project, like any other project, requires input, encouragement and assistance from countless people no matter how individual it is.

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LIST OF ABBREVIATIONS

BLAST	basic local alignment search tool
bp	base pair
BMP	bone morphogenic protein
CNTF	ciliary neurotrophic factor protein
DML	dorsal medial lip
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOMS	delayed onset muscle soreness
E-C	excitation-contraction
FGF	fibroblast growth factor
HB	homeobox
HD	homeodomain
HTH	helix-turn-helix
IGF	insulin growth factor
Kb	kilobases
MCK	muscle creatine kinase
MHC	muscle heavy chain
NC	notochord
NT	neural tube
OP	octapeptide
Pax	paired box
PCR	polymerase chain reaction

PB	paired box
PD	paired domain
Prd	Paired (*D. melangogaster*)
Q	glutamine
RNA	ribonucleic acid
SP	side population
TA	transactivation domain
UV	ultra violet
VLL	ventral lateral lip

N.B. the following conventional notation is used;

Pax indicates the gene,

Pax indicates the protein,

PAX indicates the gene from *Homo sapiens*,

PAX indicates the protein from *Homo sapiens*.

1.0 INTRODUCTION

1.1 Background to the study

Exercised-induced muscle damage is a frequent physiological phenomenon. In both humans and animals, high force eccentric muscle actions, during forcibly lengthening contracting muscle, have been found to produce pronounced muscle damage, especially if exercise is unaccustomed (in comparison to isometric and concentric muscle actions). Therefore eccentric exercise is used widely in research as a model for experimental muscle damage. A cascade of events is initiated in the muscle in order to repair damaged tissue, including degeneration and regeneration processes. Characteristic symptoms of eccentric exercise-induced muscle damage include loss of muscle strength and range of motion, swelling, increased plasma concentration of creatine kinase and myoglobin, evidenced by abnormal magnetic resonance and ultrasound images of the damaged skeletal muscle.

During muscle regeneration, satellite cells are the primary source of new myoblasts in adult muscle. These cells are located between the plasmalemma and basal lamina of mature muscle fibres. The process of muscle regeneration requires activation of quiescent mononucleated satellite cells which proliferate, differentiate and fuse together to form fully functional muscle fibres. The ability of satellite cells to self-renew ensures sufficient satellite cells for the repair of normal adult muscle throughout life. Conversely in muscle degenerative diseases, for instance Duchenne Muscular Dystrophy, the satellite cells become exhausted very quickly as a result of constant self-renewal.

Muscle stem cells (satellite cells) are specified by the gene, *Pax7*, during development. Experimental studies of *Pax7* null mice that lack the *Pax7* gene revealed that after birth there is little or no skeletal muscle growth or regeneration. The *Pax7* gene encodes a transcription factor that activates downstream target genes including those associated with muscle cell specification. Recent studies have determined polymorphic repeats within the promoter and intronic regions of the human *PAX7* gene. The promoter polymorphism has been shown to alter the transcriptional efficiency of this gene *in vitro*. It is possible therefore that polymorphisms in the gene, specifically within the promoter, alter the *in vivo* transcriptional activity of this gene and affect the ability to activate downstream target genes, including those encoding muscle proteins required for regeneration. The promoter polymorphism may have a higher impact on transcriptional activity in comparison to the intronic polymorphism, given its location and the high evolutionary conservation of the surrounding gene region. To date, a distinct link between *PAX7* polymorphisms and activation of satellite cells has not been identified.

It is interesting to note that efficiency of repair of muscle damage varies considerably between individuals; the full functional recovery after eccentric exercise-induced muscle damage varies from a few days to several weeks. Recent publications suggest newly identified polymorphisms in genes coding for some muscle proteins may influence phenotypic response to muscle damage. For example, a polymorphism in the *ciliary neurotrophic factor* gene (*CNTF*) is associated with differences in recovery of eccentric strength. Although factors have been identified that may influence muscle regeneration, there is a high possibility that variation in polymorphic sequences between individuals

may also affect skeletal muscle regeneration, by virtue of the crucial role of *PAX7* in satellite cell specification.

1.2 Significance of the study

This study provides evidence for and will assist in establishment of eccentric-exercise training programs specifically tailored to individual genetic profiles; different training programs can be developed for different types of sport with the aim of optimizing regeneration after injury, possibly increasing an individual's performance. Moreover this study provides preliminary evidence for development of clinical therapies suited for patients with muscle degenerative diseases, based on mild eccentric exercise programs that may stimulate better muscle regeneration therefore lowering disease symptoms.

1.3 Hypothesis

1. Allele frequencies of the gene, *PAX7*, differ in different population groups.
2. Allelic variation in the gene, *PAX7*, contributes to observed differences in recovery of force following damage by eccentric exercise.

1.4 Aims

The overall aims of this study are to determine allele frequencies in several population groups as well as to assess whether allelic variation in the gene, *PAX7*, can explain the observed differences in recovery of force following damage by eccentric exercise. Firstly we establish the allelic variation of *PAX7* at promoter and intron loci, within random

Australian (Caucasian), Japanese, African (Xhosa) and Chinese (Han) population groups. Thereafter we determine a statistical association between *PAX7* allelic forms and recovery of force (low or high) following eccentric exercise, indicative of muscle regenerative ability.

2.0 LITERATURE REVIEW

2.1 Muscle development

Skeletal muscles in all vertebrates derive from embryonic somites which are epithelial spheres of paraxial mesoderm (Reviewed by Asakura & Rudnicki, 2002). The formed somites differentiate and subdivide along the dorsal-ventral axis in response to signals from surrounding tissues, to form epithelial dermomyotome and mesenchymal sclerotome. The epithelial dermomyotome is the source of tissue that later develops into skeletal muscles of the trunk and limbs and the dermis of the skin. The mesenchymal sclerotome gives rise to bone, ribs and cartilage of the vertebrae (Figure 1.1) (Martini & Welch, 2001, p. 214; Brent, Schweitzer & Tabin, 2003).

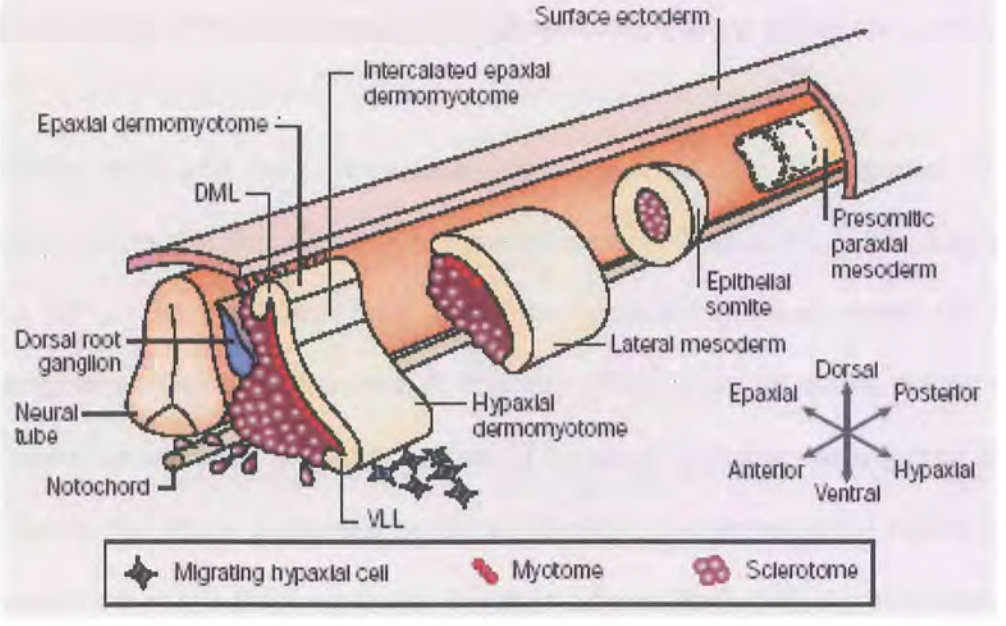


Figure 2.1: Embryonic myogenesis (Buckingham, Bajard, Chang, Daubas, Hadchouel, Meilhac, Montarras, Rocancourt & Relaix, 2003).

2.1.1 *Myogenic regulatory factors*

The epithelial dermomyotome responds to secreted signals from neighbouring tissues that increase (Wnts, Notch, BMP's) or decrease (BMP4, Shh) expression of myogenic regulatory factors MyoD and Myf5. For example, the myotome becomes muscle when it receives a signal (Wnt) from the surrounding tissue to produce MyoD protein (Rudnicki et al., 1993; Kablar et al., 1998; Reviewed by Charge & Rudnicki, 2004). In order for myogenic specification of stem cells to occur during development, many such factors must be either up regulated or down regulated at specific times, to ensure that sufficient numbers of cells remain proliferative at the same time as others are differentiating. In this way, large numbers of cells are produced to provide muscle for the whole body (Jostes, Walther & Gruss, 1990; Kawakami, Kimura-Kawakami, Nomura & Fujisawa, 1997).

In addition Pax3 and Pax7 transcription factor proteins play an important role as regulatory factors that are necessary for muscle formation (Jostes et al., 1990; Kawakami et al., 1997). The *Pax3* and *Pax7* genes are initially expressed across the entire dermomyotome (Goulding, Lumsden & Paquette, 1994). Later on during development, these genes are expressed in specific regions of the dermomyotome; experimental studies have shown that *Pax7* expression becomes intense in the dorso-medial region of the dermomyotome, while *Pax3* expression becomes reduced dorsomedially, being generally expressed in the lateral dermomyotome (Figure 2.2) (Kaehn, Jacob, Christ, Hinrichsen & Poelman, 1988; Jostes et al., 1990; Goulding et al., 1994; Tremblay et al., 1998; Chi & Epstein, 2002; Tajbakhsh, 2003). The Pax3 protein present in the lateral region of the dermomyotome activates genes that encode the two myogenic transcription factors MyoD

and Myf5. The Pax3 protein can stimulate the production of MyoD only in the absence of inhibitory transcription factors that are present in the sclerotome (Gilbert, 2000, p. 453). This specific Pax3 expression in the dermomyotome results in the formation of hypaxial myotome from Pax3 positive cells which migrate under the dermomyotome to form the ventral lateral lip (VLL). Similarly, Pax7 induces Myf and MyoD protein production in the medial region of the dermomyotome which gives rise to epaxial myotome from the cells of the dorsal medial lip (DML). Later on in development the epaxial and hypaxial myotome produce dorsal and ventral body wall musculature respectively (Figure 2.2) (Selleck & Stern, 1991; Goulding et al., 1994; Denetclaw, Christ & Ordahl, 1997; Tremblay et al., 1998; Houlzestein et al., 1999; Parker, Seale & Rudnicki, 2003).

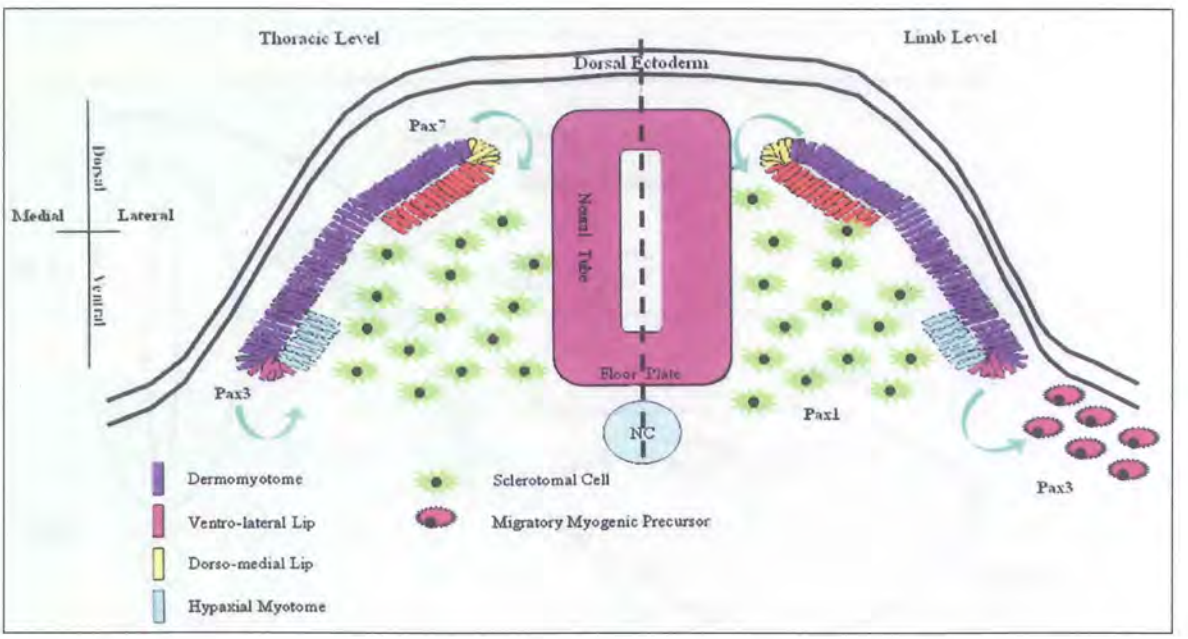


Figure 2.2: *Pax* gene expression in muscle development (Chi & Epstain, 2002).

In addition, at the region of the developing limb buds, *Pax3* expressing muscle progenitor cells of the ventral lateral lip undergo an epithelial to mesenchymal transition and delaminate (Christ & Ordahl, 1995; Bailey, Ordahl, Williams & Deneclaw, 2000; Holowacz & Lassar, 2001). The migration and delamination of cells is controlled mainly by fibroblast growth factors (FGF) and hepatocyte scatter factor (c-met). The Pax3 positive cells migrate to form muscles of the developing limbs, tongue and diaphragm (Ordahl & Le Douarin, 1992; Brand-Saberi, Wilting, Ebensperger & Christ, 1996; Heymann, Koudrova, Arnold, Koster & Braun, 1996). According to Buckingham et al. (2003) the migratory precursor cells migrate to the limb and proliferate extensively before they differentiate into muscle (Figure 2.3). The proliferation is sustained to some extent directly by Pax3 as well as by c-met.

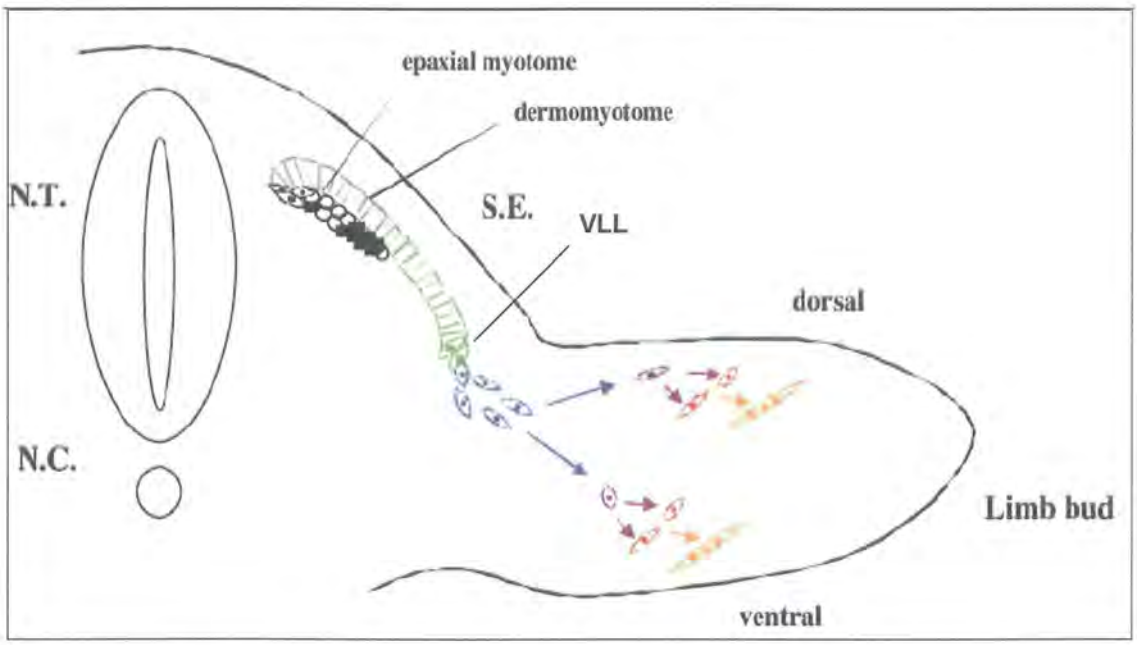


Figure 2.3: Skeletal muscle formation in the limb (Buckingham et al., 2003).

MyoD and Myf5 proteins are basic helix-loop-helix transcription factors (bHLH proteins). They have the ability to bind to similar canonical sequences/binding sites in the promoter region of muscle-specific genes. Experimental studies have shown that if the bHLH transcription factors are expressed in an undifferentiated cell of nearly any type, that cell will be assigned to a myogenic lineage (Gilbert, 2000, p. 453).

Embryonic proliferating MyoD and/or Myf5 positive muscle precursor cells are known as myoblasts. Most of the myoblasts undergo terminal differentiation and become myocytes (Figure 2.4). This is achieved by MyoD and Myf5 activating other myogenic genes encoding basic helix-loop-helix transcription factors such as Myogenin and Mef2 (Delfini, Hirsinger, Pourquie & DupreZ, 2000; Charge & Rudnicki, 2004). In addition to the transcription factor expression, the myocytes also express other muscle specific proteins such as muscle creatine kinase (MCK) and myosin heavy chain (MHC) (Hasty et al., 1993).

Upon fusion of the myocytes to form multinucleated muscle fibres, the fibres undergo maturation and become contractile units of skeletal muscle (Martini, 2001, p.274). Terminal myocyte differentiation is the arrangement of the muscle fibres into slow or fast fibres depending on the muscle function. For example, fast fibres express fast myosin heavy chains which are used for body movement. In comparison, slow fibres express slow myosin heavy chains used to maintain body posture (Miller & Stockdale, 1986; Wilmore & Evans, 2002). The patterning of the musculature is set up early in development as the muscle fibres form. The muscle arrangement in the limb is still

unclear but follows the formation of skeletal structures (Kardon, 1998; Francis-West, Antoni, & Anakwe, 2003).

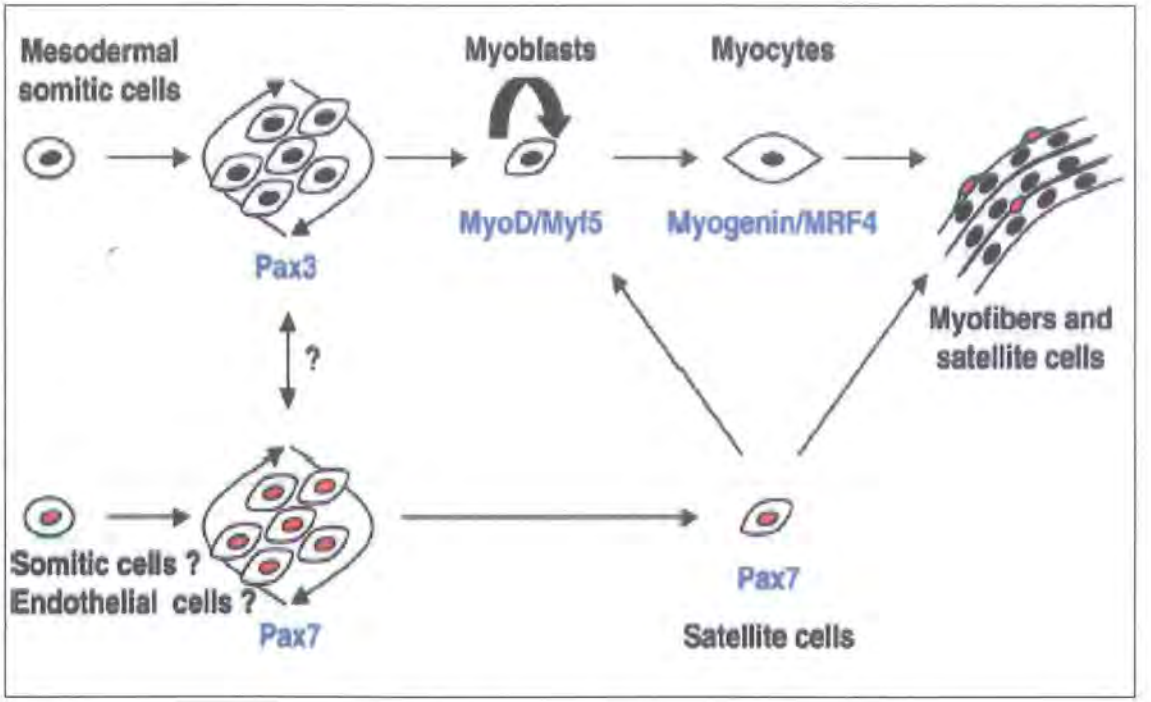


Figure 2.4: Factors and cellular events involved in muscle development (Charge & Rudnicki, 2004).

It is known that myoblast heterogeneity is an important characteristic of developing skeletal muscle. For example, during limb development both embryonic and fetal myoblasts are present. It is believed that embryonic myoblasts are the precursors of primary fibres and that fetal myoblasts are responsible for generating secondary fibres. Once the primary myofibres are formed, it is thought that there is further proliferation of fetal myoblasts to produce overlying secondary fibre (Stockdale & Miller, 1987; Harris, Duxson, Fitzsimons & Rieger, 1989; Stockdale, 1992). In view of the fact that embryonic and fetal myoblasts co-exist in the same environment, it stands to reason that they

respond differently to regulatory molecules. For example, TGF-β has no effect on embryonic myoblasts whereas it inhibits fetal myoblast differentiation (Cusella-De Angelis et al., 1994).

2.1.2 *Satellite cells in muscle development*

It is of great importance to note that not all myoblasts differentiate to become muscle fibres during development. Some myoblasts remain as undifferentiated stem cells throughout life. These cells are known as satellite cells by virtue of their geographical location between the plasmalemma and basal lamina of muscle fibres, in adult skeletal muscle (Martni, 2001, pp. 273-274). The developmental origin of satellite cells remained contraversial until recently. Early experiments with quail-chick chimaeras by Armand, Boutineau, Mauger, Pautou & Kieny (1983) indicated that satellite cells may derive from somites. By contrast, later research led to the hypothesis that satellite cells arise from endothelial cells of the dorsal aorta, since these cells produce muscle precursor cells in response to signals from neighboring muscles (De Angelis et al. 1999). However, very recent evidence proves conclusively that satellite cells arise form somites (Relaix, Rocancourt, Mansouri & Buckingham, 2005). It has been suggested that in developing limb, some myoblasts stay as satellite cells which are responsive to cues that prevent differentiation (evidence such as the presence of TGF-β receptors support this) (Cusella-De Angelis et al., 1994).

Satellite cells express *Pax7* and may arise from *Pax7* expressing cells of the dermomyotome (Relaix et al., 2005). While satellite cells are thought to be specified

during development by *Pax7*, *Pax7* also remains expressed in satellite cells throughout life (Cornelison & Wold, 1997; Grounds, 1999; Seale & Rudnicki, 2000; Hawke & Garry, 2001). Notably, *Pax7* null mice show an almost complete absence of satellite cells in mature skeletal muscle fibres (a few Pax3 positive satellite cells are seen in specific muscles) (Mansouri, Stoykova, Torres & Gruss, 1996, Buckingham et al., 2003; Kassar-Duchossoy et al., 2005).

The number of satellite cells present in normal adult muscle varies depending on the muscle age and muscle group (Sabourin & Rudnicki, 2000). When activated, a satellite cell will produce each round of cell division, two different daughter cells by asymmetric cell division (Moss & Leblond, 1971; Snow, 1977; Goldring, Patridge & Watt, 2002), one daughter cell remains as a satellite cell, whereas the other daughter cell differentiates along a myogenic lineage. There is a limit to the number of mitotic divisions that a satellite cell can undergo. In normal skeletal muscle, the number of cell divisions the cell undergoes is sufficient to provide satellite cells for the duration of the life of the muscle. However, in the muscles of patients suffering from degenerative muscle disease, such as Duchenne Muscular Dystrophy, the satellite cells become exhausted very quickly because of continuous cell divisions (Bulfield, Siller, Wight & Moore, 1984; Webster & Blau, 1990).

2.2 Muscle regeneration

Muscle regeneration is an essential process for the preservation of muscle function. Adult skeletal muscle has the ability to regenerate in response to severe damage. For example, muscle regeneration will take place where muscle is traumatised (resistance training) as well as in the case of degenerative muscle disease. Regeneration consists of two components, degeneration followed by regeneration of injured muscle tissue (Figure 2.5) (Charge & Rudnicki, 2004).

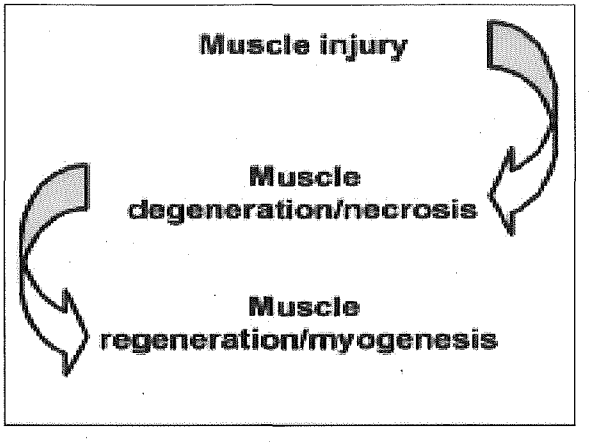


Figure 2.5: Degenerative and regenerative phases in skeletal muscle repair (Charge & Rudnicki, 2004).

2.2.1 *Degeneration of injured muscle tissue*

The degeneration component of muscle regeneration starts with necrosis of injured muscle tissue. This process is initiated by destruction of the myofibril membrane resulting in increased permeability, induced by creatine kinase and other muscle proteins showing increased blood serum levels after muscle injury (Reviewed by Armstrong, Warren, & Warren, 1991). For the muscle cell to begin regeneration, the necrotic tissue

must be removed from the site of injury by the process of phagocytosis. This is achieved by macrophages and polymorphonuclear leukocytes (neutrophils) that travel via the blood vessels to the site of necrotic muscle fibres (Reviewed by Grounds, 1991). The damaged region of the muscle is sealed off in order to decrease degeneration of other parts of the muscle (Hurme & Kalimo, 1992).

2.2.2 *Regeneration of injured muscle tissue*

Muscle degeneration is followed by muscle regeneration. In this phase, satellite cells become activated; they exit from an inactive state and start proliferating (Grounds, 1991; Miller, Scaefer, & Dominov, 1999). Growth factors released from macrophages and endothelial cells (eg. Fibroblast growth factor), bind to membrane receptors and activate satellite cell proliferation leading to growth of the muscle (Figure 2.6) (Grounds, 1991). Competence and progression factors are two types of growth factors that are involved in muscle development and repair. The former types of factors have an effect on the satellite cells by moving them from G0 to G1 phase (eg. platelet derived growth factors). On the other hand, progression factors have an effect on satellite cells by moving them from G1 to the DNA synthesis, S phase of the cell cycle (eg. Insulin growth factors IGF-I and IGF-2) (Grounds, 1991; Chambers & McDermott, 1996). As the myoblasts begin to differentiate *Pax7* expression becomes down-regulated (Cornelison & Wold, 1997; Grounds, 1999; Seale & Rudnicki, 2000; Hawke & Garry, 2001). After differentiation, the myocytes fuse together and form new muscle fibres to replace those that are damaged (Figure 2.7) (Parker et al., 2003; Charge & Rudnicki, 2004).

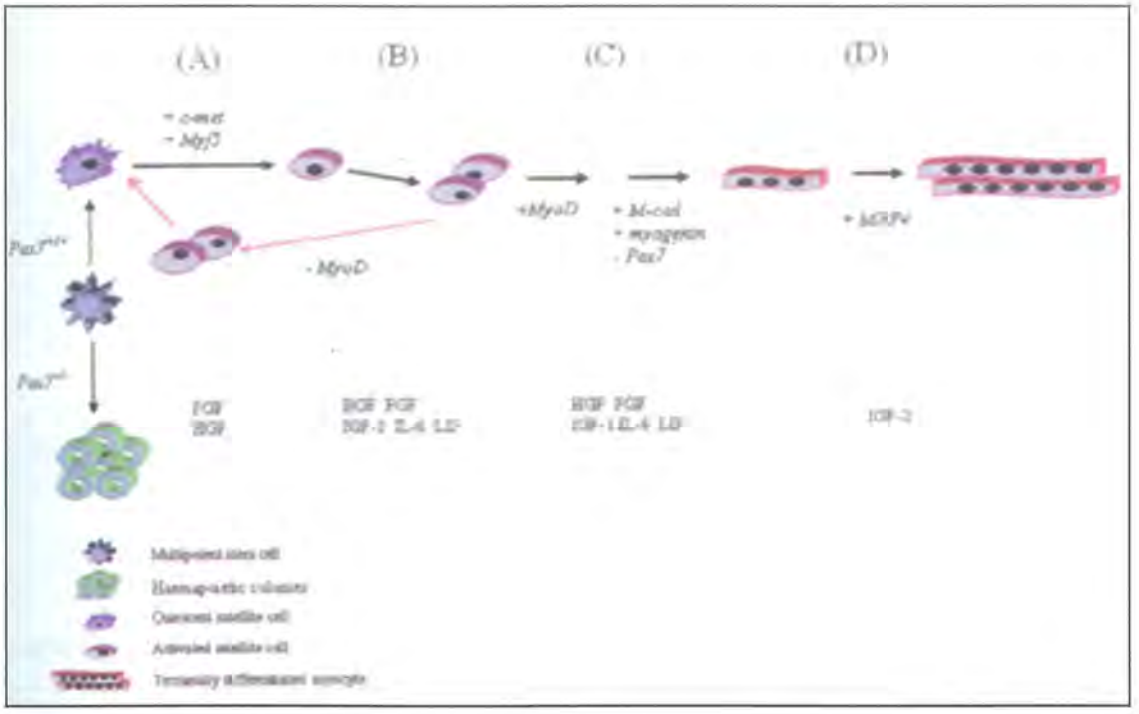


Figure 2.6: Growth factors involved in myogenesis (Lamey, Koenders & Ziman, 2004).

Recent experiments have shown that in addition to the satellite cell contribution to muscle regeneration, there are adult stem cells in skeletal muscle tissue that may be involved in muscle regeneration. Side-population cells (SP), for example can play a minor role in regeneration as well as in production of satellite cells (Gussoni, Soneoka, Strickland, Buzney & Khan, 1999; Asakura, Seale, Girgis-Gabardo & Rudnicki, 2002; Musaro et al., 2004). In addition, experimental studies have shown that stem cells isolated from bone marrow also have the potential to become satellite cells (Ferrari et al. 1998). Interestingly, Pax7 has a strong involvement in stem cell mediated muscle repair; Pax7 is expressed in stem cells that differentiate along a myogenic lineage (Figure 2.7) (Musaro et al., 2004).

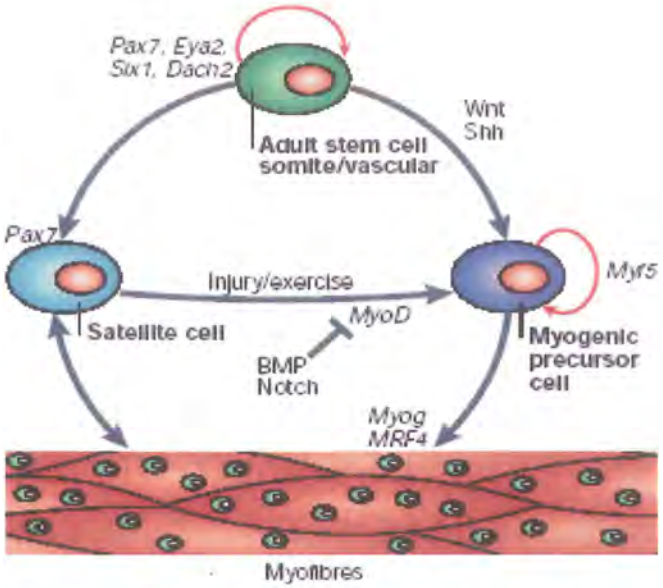


Figure 2.7: Skeletal muscle regeneration after injury (Parker et al., 2003).

2.3 Exercise-induced muscle damage

Muscle damage induced by exercise is predominantly induced by an overload stimulus in order to generate training adaptation. The factors that may contribute to overload include training volume, duration, frequency and intensity of exercise. In the process of generating training adaptation, physiological pain takes place as some of the tissue becomes damaged or injured (Nosaka, Lavender, Newton & Sacco, 2003). There are three main types of exercise induced muscle injury (based on clinical presentation). The first type includes delayed onset muscle soreness (DOMS) that arises 24 to 48 hours after reaching the overload stimulus. The second type of muscle injury involves a tear of a few muscle fibres or a tear of muscle fibres and fascia resulting in severe pain. The last type

of muscle injury is evident during or after exercise and presents as cramps and/or soreness (Safran, Seaber, & Garrett, 1989).

2.3.1 *Eccentric exercise and muscle damage*

Concentric muscle action is used to initiate body movements and involves shortening of muscle. By contrast, eccentric muscle action is used to slow or stop movements and involves forcibly lengthening contracting muscle (Proske & Morgan, 2001). It is known that in comparison to isometric and concentric exercises, eccentric exercise produces greater skeletal muscle damage, particularly if it's unaccustomed (McCully & Faulkner, 1985; Clarkson, Nosaka & Braun, 1992; Gibala et al., 2000). It is believed that eccentric exercise-induced muscle damage is responsible for the development of delayed onset muscle soreness (Nosaka et al., 2003). However delayed onset muscle soreness cannot be used to indicate extent of muscle damage (Nosaka, Newton & Sacco, 2002). Also it is important to note that the amount of damage is dependent on muscle length; for example, there is more muscle damage if eccentric exercise is performed on a descending limb in comparison to an ascending limb (Nosaka & Sakamoto, 2001).

2.3.2 *Symptoms of eccentric exercise-induced muscle damage*

Symptoms of eccentric exercise-induced muscle damage include: swelling, abnormal magnetic resonance and ultrasound images, loss of muscle strength and range of motion, and increased plasma concentration of muscle proteins (creatine kinase, myoglobin) (Figure 2.8) (Clarkson et al., 1992; Howell, Chleboun, & Conaster 1993; Nosaka & Clarkson, 1996; Sorichter et al., 2001). Much of the damage is initiated by disruption of

the sarcomeres and the excitation-contraction (E-C) coupling system (Proske & Morgan, 2001). Additionally, changes in proprioception and muscle sense organ damage, result in a change in optimum length for active muscle tension, together with a decrease in active tension and an increase in passive tension (Reviewed by Proske & Morgan, 2001). Homeostatic intracellular calcium levels are modified considerably because of damage to the plasma membrane. This in turn causes further degeneration of plasma membrane and muscle fibres (Armstrong et al., 1991). Damage to the muscle fibres causes activation of an inflammatory response, infiltration of macrophages and degradation of damaged proteins (Pyne, 1994; Stauber & Smith, 1998; Clarkson & Sayers, 1999). Secretion of growth factors and cytokines from inflammatory cells ultimately leads to activation of satellite cells and regeneration (via satellite cell activation) of damaged muscle cells and restoration of muscle function (Hawke & Garry, 2001).

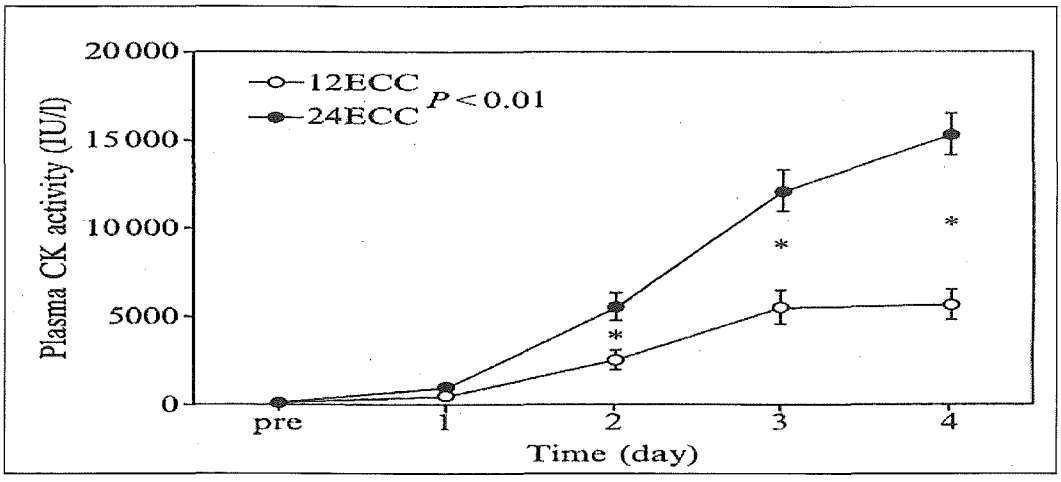


Figure 2.8: Plasma CK activity before and after eccentric exercise (Nosaka et al., 2002).

2.3.3 *Effects of eccentric exercise*

While eccentric exercise causes more muscle damage than other forms of exercise (McCully & Faulkner, 1985; Gibala et al., 2000), it is well documented as being beneficial for expansion of muscle size and strength (Figure 2.9). For example, a study has shown that a 12 week period of isokinetic eccentric training on knee extensors resulted in enlargement of type IIa fibres whereas isokinetic concentric training caused little change in fibre size (Hortbagyi et al., 1996; Nosaka et al., 2003). It is interesting to note that an initial session of eccentric exercise will protect against further damage caused by a second comparable bout of exercise (Clarkson et al., 1992; Clarkson & Sayers, 1999; McHugh, Connolly, Eston, & Gleim, 1999). There is not enough data to explain this muscle adaptation but it is suggested that neural tissue, connective tissue and cellular adaptations play an important role (Clarkson & Sayers, 1999; McHugh et al., 1999). In addition, satellite cell adaptations may play a role. The protective effect on eccentric exercise-induced muscle damage lasts several weeks or more depending on the severity of eccentric exercise (Clarkson et al., 1992; Nosaka, Newton & Sacco, 2001).

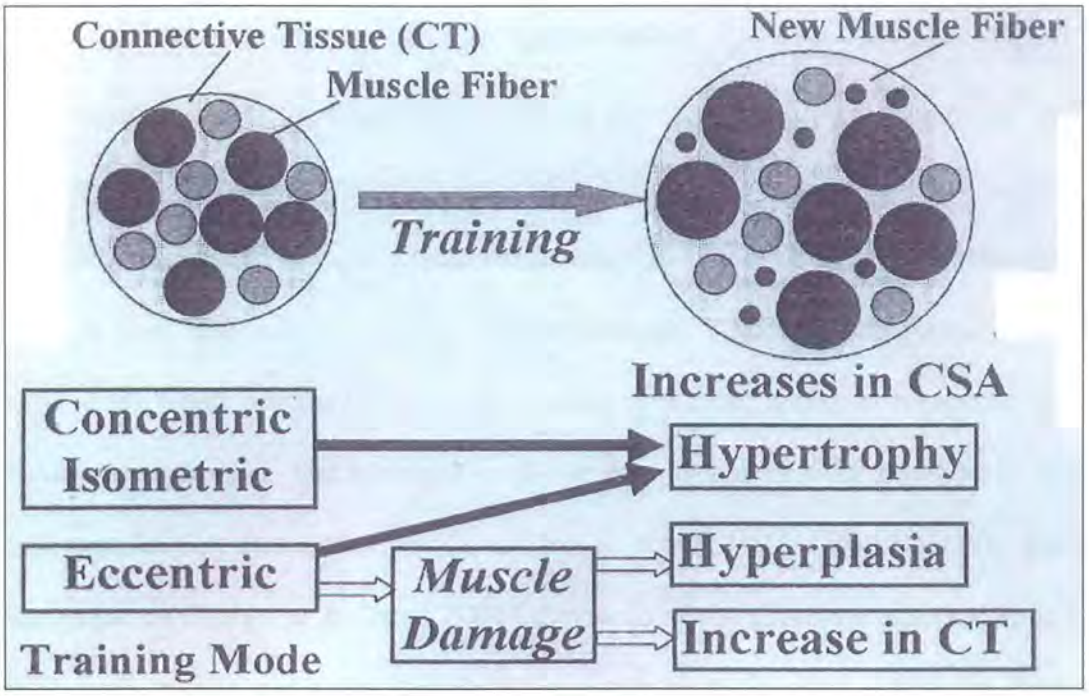


Figure 2.9: Increase in muscle cross-sectional area after concentric, isometric and eccentric training (Nosaka et al., 2003).

2.3.4 *The adaptation effect and possible treatments*

The adaptation effect from mild eccentric muscle actions can be used to reduce muscle damage from competitive sports such as football and rugby which usually put great demands on muscles (Proske & Morgan, 2001). The muscle adaptation effect can be very beneficial in treating degenerative muscle diseases. For example, this type of treatment can be used to treat Duchenne Muscular Dystrophy; a very mild eccentric exercise program will not further damage already affected muscle and there is a possibility that this type of eccentric exercise could play a role in effecting the adaptation process and result in some preservation of muscle tissue and function (Proske & Morgan, 2001).

2.3.5 *Variation in skeletal muscle regeneration*

Of particular note is the large variation in an individual's ability to regenerate skeletal muscle. Quantification of muscle regenerative ability (by assessing range of motion and plasma concentration of muscle proteins) reveals that an individual may be classified as a good or poor responder to exercise induced damage. While the importance of satellite cells in the repair process is known, the causes of varying ability to regenerate skeletal muscle are unknown. The transcription factor Pax7 is known to be involved in satellite cell specification and activation (Cornelison & Wold, 1997; Grounds, 1999; Seale & Rudnicki, 2000; Hawke & Garry, 2001). Evidence of the important association of Pax7 with satellite cells and muscle regeneration comes from observed deficits in *Pax7* null mice. Skeletal muscle of *Pax7* null mice is lacking in satellite cells. The muscles, while normal in structure, are much smaller than those of wild type mice, due to the absence of satellite cells for muscle growth and regeneration (Mansouri et al., 1996). It appears certain then, that genetic differences such as those in *Pax7* may play a role in adaptation/response to muscle damage. In this project the association between *Pax7* allelic forms and regenerative ability will be assessed.

2.4 *Pax7* gene structure and function

2.4.1 *Pax7 genomic and protein structure*

Pax7 is a member of the *Pax* gene family which encode highly conserved DNA binding proteins (transcription factors). Research studies have shown that these transcription factors are crucial regulators of developmental biology in all complex organisms (Reviewed by Chi & Epstein, 2002). The *Pax7* family member is particularly associated with myogenesis and neurogenesis during development (Jostes, Walther, & Gruss, 1991). The *Pax7* gene contains a paired-box (PB) that encodes the conserved paired DNA binding domain and a paired-type homeobox (HB) encoding the homeodomain, also a conserved DNA binding domain. In addition, within *Pax7* there are the conserved octapeptide (OP) and the C-terminal transactivation domain (TA) encoding regions (Figures 2.10 & 2.11) (Balczarek, Lai & Kumar, 1997). Within the encoded paired domain, there are two sub-domains and each sub-domain is characterized by a helix-turn-helix (HTH) motif as is the homeodomain (Treisman, Harris & Desplan, 1991; Schafer, Czerny, Bernasconi, Genini & Busslinger, 1994; Xu et al., 1999; Ziman, Rodger, Chen, Papadimitriou, Dunlop & Beazley, 2001).

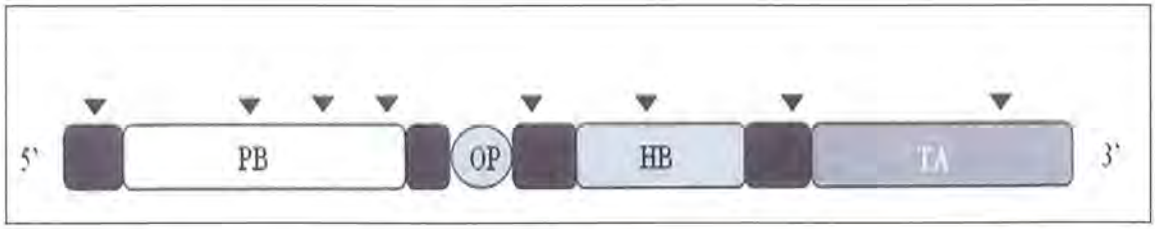


Figure 2.10: *Pax7* genomic structure and regions encoding protein domains. The arrows indicate exon/intron boundaries. PB = paired box encoding region, OP = octapeptide encoding region, HB = homeobox encoding region and TA = transactivation domain encoding region (Chi & Epstein, 2002).

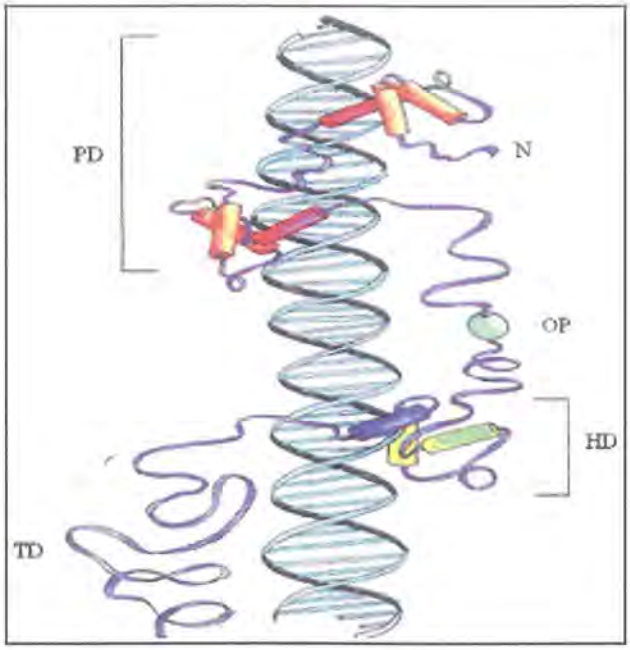


Figure 2.11: Pax7 protein structure, shown bound to chromosomal DNA. PD = paired domain, HD = homeodomain, OP = octapeptide, TD = transactivation domain, N = N terminus and C = C terminus (Chi & Epstein, 2002).

The human *PAX7* gene is mapped to chromosome 1p36.2 (Vorobyov, Mertsalov, Dockhorn-Dworniczak, Dworniczak & Horst, 1997). Other genes mapped to this same chromosomal region include: *TNFR2*, tumor necrosis factor receptor; *ID3,* which encodes a helix-loop-helix protein; *CDC2L1*, which encodes a cell-regulated kinase; *DAN*, encoding a transcription factor and *E2F-2*, a transcription regulator (White et al., 1995). It is important to note that this chromosomal region is rearranged or deleted frequently in cancers, placing the *PAX7* gene as a potential candidate for such disorders as neuroblastoma, breast cancer, leiomyoma, melanoma and pheochromocytoma (Report of the Second International Workshop on Human Chromosome 1 Mapping, 1996; Vorobyov et al., 1997). The coding region of human *PAX7* may vary due to alternate splicing and consists of eight (*PAX7A*) or nine (*PAX7B*) exons disrupted by seven or eight introns respectively (Barr et al., 1999). Studies have shown that human and mouse *PAX7/Pax7*

are very similar in structure and sequence. For example, at the amino acid level, the paired domains and the octapeptides are 100% identical (Burri, Tromvoukis, Bopp, Frigerio & Noll, 1989; Shapiro et al., 1993; Lamey, et al., 2004).

2.4.2 *Pax7 function*

Pax7 and other Pax proteins are thought to function by binding to specific DNA sequences, generally enhancer regions in the promoters of downstream target genes, leading to alteration of transcription of bound target genes. The function of the conserved paired domain was initially identified as a DNA binding domain in the *Drosophila* paired protein, Prd (Underhill & Gross, 1997; Plaza et al., 2001). The paired domain has an important role in DNA recognition as does the homeodomain which also may assist in co-operative homodimer or heterodimer formation on longer DNA consensus sequences (Schafer et al., 1994). In addition current experimental evidence shows that the transcriptional activity can be modified as a result of intramolecular interactions among different binding domains (Underhill & Gross, 1997).

2.4.3 *Alternate Pax7 transcripts*

It has been shown that alternate gene transcripts of several *Pax* genes are responsible for their different biological functions. Because the *Pax* genes are involved in development of different organs (brain, muscle etc.), the different transcripts may differentially control development of different organs (Poleev et al., 1992; Kozmik, Kurzbauer, Dorfler & Busslinger, 1993; Tsukamoto, Nakamura & Niikawa, 1994; Stoykova & Gruss, 1994). The *Pax7* paired box transcripts have been discovered in zebrafish, mouse and human

(Vorobyov et al., 1997; Seo, Saetre, Havik, Ellingsen & Fjose, 1998; Ziman & Kay, 1998; Barr et al., 1999; Seale et al., 2000). Four alternate *Pax7* transcripts have been identified; *Pax7a, Pax7b, Pax7c,* and *Pax7d* (Figure 2.12). These transcripts differ from each other by inclusion or exclusion of a trinucleotide (CAG) or a hexanucleotide (GTTTAG) resulting in transcripts with the inclusion of a glutamine (Q) in the linker region and a glucine-leucine (GL) in the second HTH of the paired domain (Vogan, Underhill, & Gros, 1996; Kay & Ziman & Kay, 1998; Ziman et al., 2001).

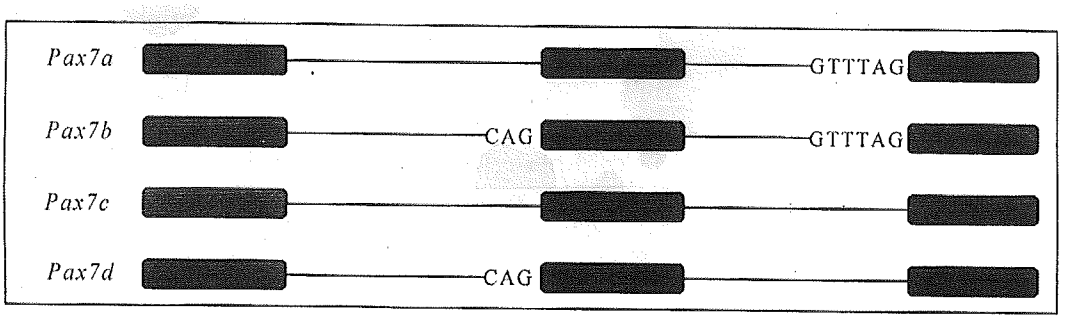


Figure 2.12: *Pax7* alternate transcripts (Ziman et al., 2001).

In addition *Pax7* transcripts can occur with distinct 3' ends (Vogan et al., 1996) (as mentioned earlier). For example, the human *PAX7A* transcript contains eight exons whereas *PAX7B* contains nine exons (Barr et al., 1999). While the murine *Pax7A* and *Pax7B* transcripts contain the same number of exons as the human transcripts, there are some significant differences in the encoded C-termini; whereas the PAX7B/Pax7B isoforms have identical C-termini, there is only 7% homology in the C-terminus of murine *Pax7A* relative to human *PAX7A* (Barr et al., 1999; Lamey et al., 2004).

Previous studies argue that different intronic structures have an effect on splicing leading to production of different populations of transcripts. The different transcripts may be produced at different rates because the splice sites are not all equally optimal for the spliceosome machinery (Libri, Goux-Pelletan, Brody & Fiszman, 1990; Krawczak, Reiss & Cooper, 1992; Luukkonen & Seraphin, 1997). Different quantities of the alternate transcripts are produced at different times in development and in different tissues (Kay & Ziman & Kay, 1998; Lamey et al., 2004-personal communication). It is possible that different polymorphisms within genes may affect splicing, thus producing different transcript profiles (Kreivi & Lamond, 1996). Further experimentation is required to investigate the association between polymorphisms and splice site selection.

2.5 Allelic forms of human *PAX7*

2.5.1 *PAX7 intron-associated polymorphism*

A polymorphic site is known to be present within the second intron of the paired box of human *PAX7*. To date, three allelic forms have been identified. These allelic forms differ in the number of tandem tetranucleotide (GAAG) repeats at the polymorphic site in the second paired box intron. In addition to this, within the same intron another polymorphic site has been identified which includes variable dinucleotide (TG) repeats (Figure 2.13). For example, *PAX7(I)* allele contains 13 tetranucleotide repeats and up to 15 dinucleotide repeats (Ziman, Pelham, Mastaglia & Kay, 2000). From previous studies it can be

concluded that the polymorphisms are inherited in a Mendelian fashion indicating that they are linked. Evidence also indicates that the allelic forms occur in an altered frequency in patients with dermatomyositis and myopathy which signifies that *PAX7* may play an important role in neuromuscular disorder development (Ziman et al., 2000). However a distinct link between these polymorphisms and activation of satellite cells has not been identified.

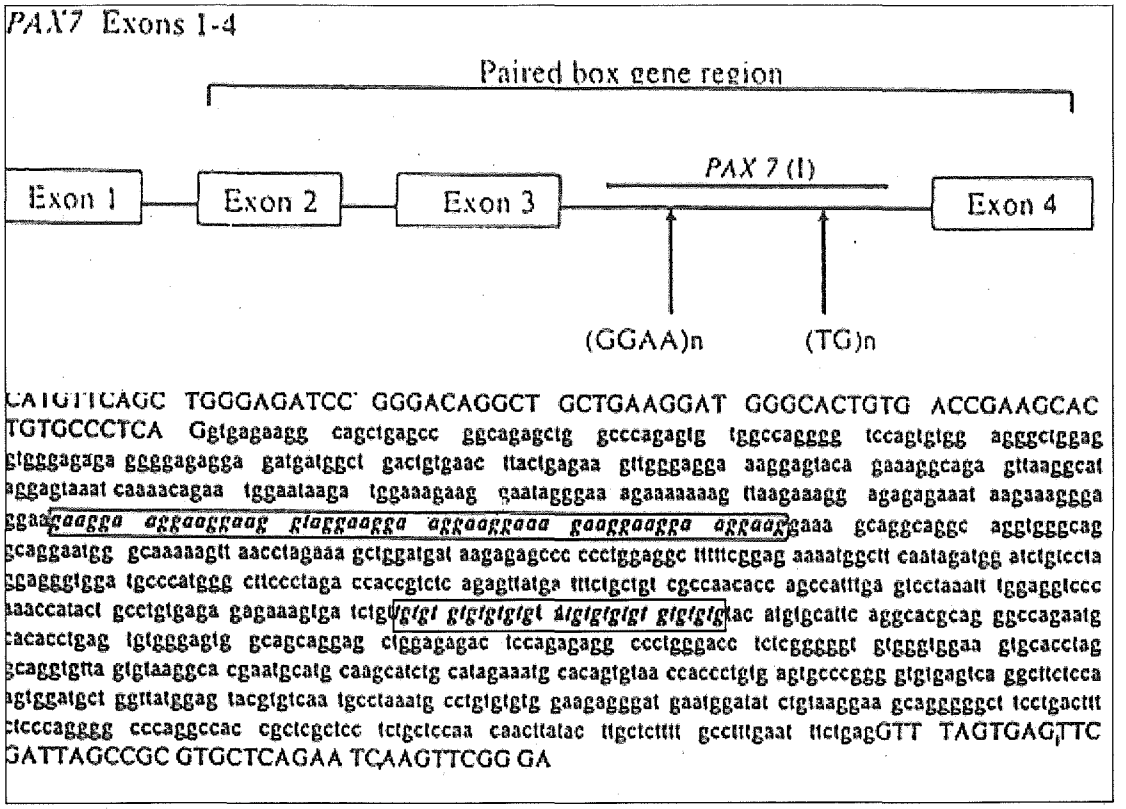


Figure 2.13: Intronic polymorphism of the *PAX7* gene (Ziman et al., 2000).

2.5.2 *Polymorphism in the promoter of PAX7*

The promoter region of each gene controls expression of that gene and therefore it is of great importance. The promoter is the nucleotide sequence located upstream (or 5') of the coding region of a gene (Jorde, Carey, Bamshad & White, 2000, p. 11). The promoter consists of a core promoter containing a TATA box where RNA polymerase II binds to initiate transcription of the gene. In addition, the promoter may contain enhancer or silencer sequences close to or thousands of bases away from the core promoter (Jorde et al., 2000, pp. 13-14).

There are more than fifty different proteins involved in the initiation of gene transcription. Some of these proteins include transcription factors that are part of the RNA polymerase II complex, necessary for transcription of DNA to mRNA. The important thing about these transcription factors is that they each bind to a specific DNA sequence in the promoter region of the gene. For example, proteins TFIID etc. bind to the TATA transcription initiation sequence. The specific binding to DNA is achieved by DNA binding motifs (Jorde et al., 2000, pp. 13-14). For Pax7, these include a paired domain containing a helix-turn-helix motif, the third α-helix of which binds to the major groove of DNA (Treisman et al., 1991; Schafer, Czerny et al., 1994; Xu et al., 1999; Jorde et al., 2000 p. 15; Ziman et al., 2001).

The basal rate of transcription is regulated by enhancer and silencer sequences in the promoter region. When the transcription factors (activators) bind to the enhancer sequences they will also bind to co-activators, a group of proteins that link the activator

protein to the binding protein and RNA polymerase at the core promoter. Transcription factors that bind to the enhancer sequences will increase the transcriptional activity and thus increase gene expression by causing the unwinding of the DNA molecule (Figure 2.14). On the other hand, if transcription factors bind to silencer sequences and form a complex of proteins, a reduction in gene expression results (Jorde et al., 2000, p. 14).

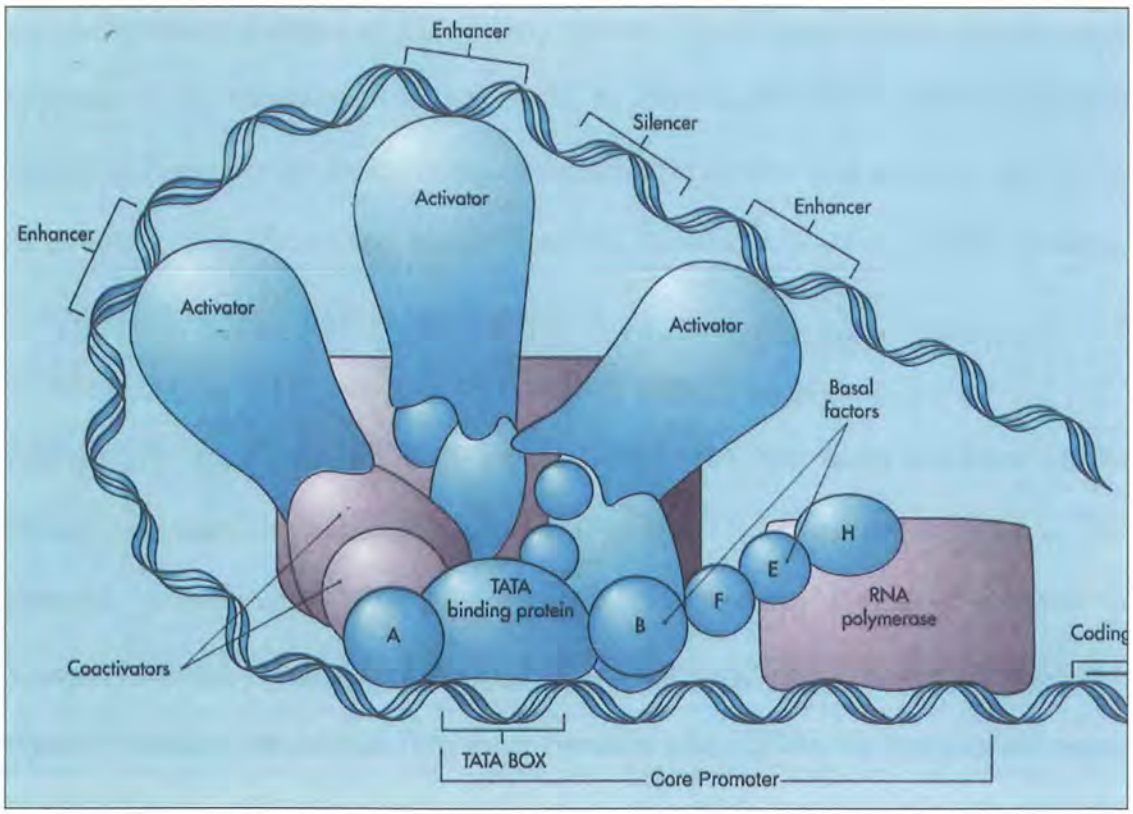


Figure 2.14: Transcription control elements (Jorde et al., 2000, p. 14), showing transcription factors bound to core promoter or enhancer or silencer sequences as well as to each other to form a transcription factor complex that regulates the rate of transcription by RNA polymerase II.

The rate or regulation of expression of genes can be altered by mutations/polymorphisms in the core promoter, or in enhancer or silencer sequences. Such changes to these important sequences could lead to aberrant gene expression and result in changes in

cellular processes (Jorde et al., 2000, p. 14; Gilbert, 2000, p. 119). For *PAX7*, polymorphisms in the promoter region could translate into an improvement or reduction in the ability to repair skeletal muscle.

Recent studies have identified and characterized the promoter region of human *PAX7*. The transcription start site is located 664 base pairs upstream of the ATG codon. The TATA-like motif is present at -177 and an inverted CCAAT box is present 222 base pairs upstream of the transcription initiation site. In addition, the *PAX7* promoter contains consensus sequences for several common transcription factors, such as *Oct-1, NF1, AP2, AP4, CREB, Sp1, Nkx2.5* and *MyoD* (Syagailo, Okladnova, Reimer, Grable, Mossner, Gattenlohner, Marax, Meyer & Lesch, 2002).

Interestingly, a polymorphic CCT repeat located 107 base pairs upstream of the transcription start site has been identified (Figure 2.15). Three allelic forms of this promoter polymorphism have been identified in unrelated Caucasian samples of European descent. Allele 8 contains eight repeat units and has a frequency of 0.38 in a random Caucasian population. The most common allele, allele 10, contains ten repeat units with a frequency of 0.59, whereas the least common allele, allele 11, contains eleven repeats and has a frequency of 0.03 in a random Caucasian population (Syagailo et al., 2002).

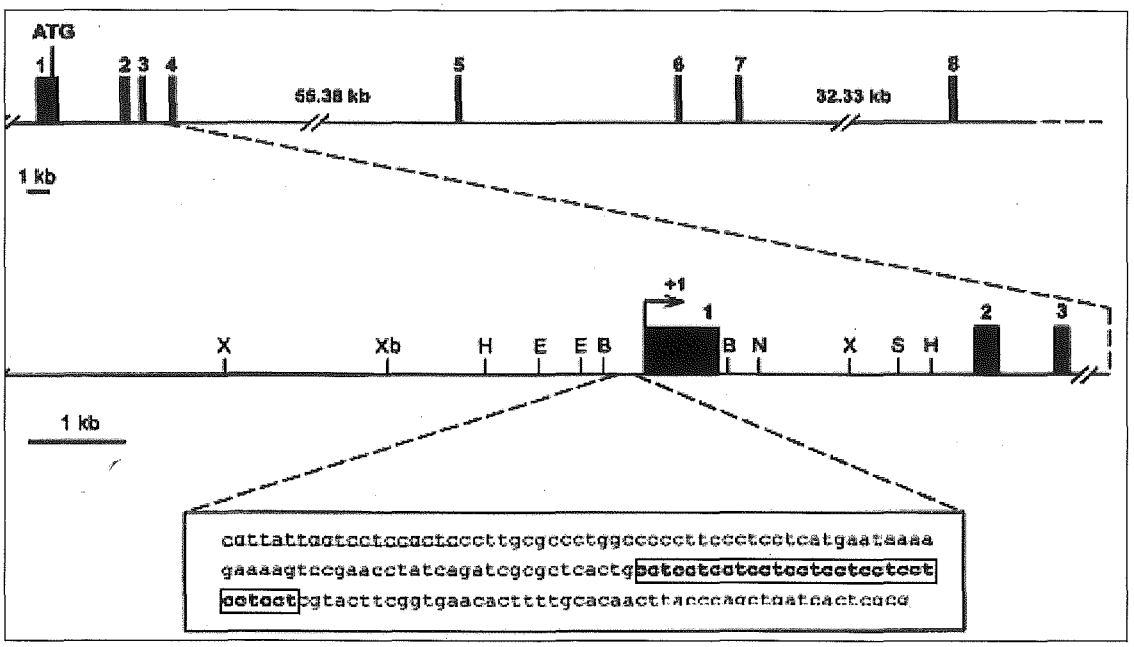


Figure 2.15: Promoter polymorphism (CCT repeat) in the *PAX7* gene (Syagailo, et al., 2002). Allele 10 is shown.

Most significantly, this study by Syagailo et al., 2002 has verified that the promoter polymorphism has an effect on transcriptional efficiency. For example, using *in vitro* promoter construct studies, the least common allele 11 was shown to have higher transcriptional efficiency when present in the *PAX7* promoter compared to alleles 8 and 10 (Syagailo et al., 2002). To date, no other studies have investigated the effects of the promoter-associated polymorphism on *PAX7* function. Importantly, it is not known as yet whether the length of the repeat-polymorphism in the promoter influences transcriptional efficiency *in vivo*. Therefore in the study presented here, we have assessed the frequency of this promoter polymorphism in different populations including non-Caucasian populations and assessed its association with muscle regeneration.

2.5.3 *PAX6 promoter-associated polymorphism*

Polymorphic repeats within a promoter region have been shown to be involved in regulation of *PAX6* gene expression. *PAX6* expression is controlled by two promoters A and B. It is reported that in the B type promoter, approximately 1 kb upstream of the transcription start site, a dinucleotide repeat has been found (AC)m(AG)n (Figure 2.16) (Okladnova, Syagailo, Tranitz, Stober, Riederer, Mossner & Lesch, 1998). 65% of Caucasian individuals are heterozygous for this polymorphism. Similar to that observed for *PAX7*, the *PAX6* alternate allelic forms have different transcriptional efficiencies. For example, the *PAX6* alleles that have more than 29 repeats has a transcriptional activity 4-9 fold greater than the allele containing 26 repeats. It was concluded that promoter associated polymorphic repeats play an important role in *PAX6* expression and therefore may be responsible for individual differences in brain function (Okladnova et al., 1998).

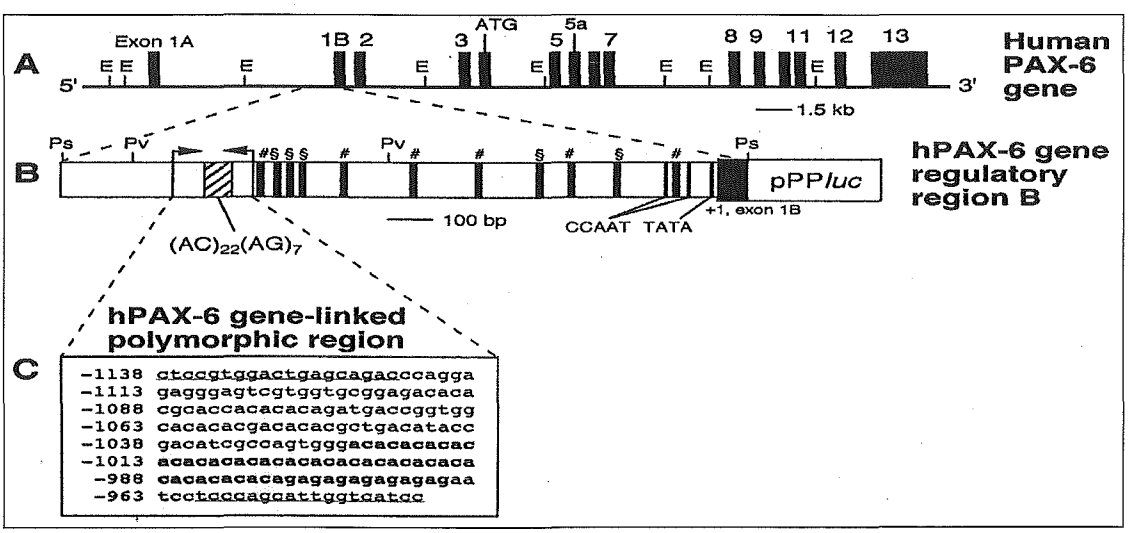


Figure 2.16: Promoter polymorphism in the human *PAX6* gene (Okladnova et al., 1998).

Conclusion

From a review of the literature it is apparent that individuals vary in their rate of repair of eccentric exercise-induced muscle damage. Recent publications suggest that polymorphisms in genes coding for two myofibrollar proteins may influence phenotypic responses to muscle damage. Our research focused on polymorphic variation in the highly conserved transcription factor, PAX7, which is expressed during development of skeletal muscle. PAX7, is involved in specification of satellite cells and it is responsible for maintaining the population of stem cells, satellite cells in adult muscle. Polymorphisms in the *PAX7* gene particularly in the promoter region, may influence phenotypic response to muscle damage. Both promoter and intron associated polymorphisms of *PAX7* may play an important role in determining the amount of *PAX7*, the number of satellite cells, or transcription profiles. Therefore, in this project we assess the association of *PAX7* allelic forms with regenerative ability, a measure of functional PAX7 activity.

3.0 MATERIALS AND METHODS

3.1 Subjects

Four different population groups were used to classify allelic forms of *PAX7* at two different polymorphic loci. Forty Australian (Caucasian), fifty Japanese and thirty two Chinese (Han) subjects were genotyped with respect to the promoter polymorphism. Forty Australian (Caucasian), forty five Japanese, thirty Chinese (Han) and thirty African (Xhosa) subjects were genotyped with respect to the intron three polymorphism. In addition, eleven Australian (Caucasian) and twenty Japanese subjects were genotyped with respect to both polymorphisms and were required to complete the exercise component of the study. A three generation family of Australian subjects were genotyped to confirm the heritable nature of the alternate *PAX7* allelic forms.

All subjects (both exercised and non-exercised) were required to provide a small sample of buccal cells or blood from which DNA was extracted and used to classify allelic forms of *PAX7*. An information sheet was given to all subjects and they were required to sign a written informed consent form, consistent with ECU Human ethical standards. The subjects were asked some questions about their country of origin to assess their heritage and genetic background. Those participants completing the exercise component of the study were graded as 'low' or 'high' responders based upon their rate of recovery of torque to pre-exercise levels. After collection of the samples, all samples were identified by number only. Access to identity details were available to chief investigators only and were stored in locked filing cabinets. Ethics approval for this project (code-04193) has been given by the Human Research Ethics Committee, Edith Cowan University, Perth, W.A.

3.2 Exercise-induced muscle damage

The exercise component of the study was performed on Australian and Japanese subjects by Associate Professor Ken Nosaka and Mr Mike Newton, Edith Cowan University, Perth, W.A.

3.2.1 *Protocol to induce muscle damage*

Individuals performed ten sets of six repetitions of eccentric contractions of the elbow flexor muscles on a Cybex 6000 isokinetic dynamometer at a velocity of 90 degrees per second, with the exercised arm moving from a starting point of 60 degrees of full flexion through a range of motion of 120 degrees. Following each repetition the arm was passively returned to the starting position at 9 degrees per second. The exercised arm was randomly assigned between subjects and counterbalanced for arm dominance. Each exercise set was separated by 3-minute intervals (Zainuddin, Newton, Sacco, & Noska, 2005).

3.2.2 *Criterion measures used to assess muscle damage and rate of functional recovery*

Measurements were taken before, immediately post, and 1, 2, 3, 4, 5, 6, & 7 days following the exercise task. 30 μl of blood was taken from a lancet puncture of a finger at time points indicated and analysed spectrophotometrically for Creatine Kinase (CK) levels. Maximal isometric torque (MIT) produced at a fixed elbow joint angle of 90 degrees was measured at the time points specified above. Range of motion (ROM) as a measure of rate recovery was determined by the difference between the fixed (FANG)

and stretched (SANG) elbow joint angle, as measured by goniometry, at time points specified above. Arm circumference (CIR) for tracing recovery was determined by a tape measure at 3, 5, 7, 9, 11 cm above the elbow on the relaxed upper arm and was used as an indicator of muscle swelling at indicated time points (Zainuddin et al., 2005).

Associate Professor Ken Nosaka and Mr Mike Newton consider that it is reasonable to sub-divide the study population into 'low' or 'high' responders. As indicated in their own and others' publications (Nosaka, Sakamoto, Newton & Sacco, 2001; Nosaka & Newton, 2002) a recovery rate (RR) was calculated as a percentage measure using the following calculation:

$$\mathbf{RR} = \frac{\textbf{Recovery force (day 5) - Recovery force (day1)}}{\textbf{Baseline force (pre) - Recovery force (day 1)}} \times \mathbf{100}$$

Subjects that produced recovery rates below 40% were considered as poor responders and subjects with rates greater than 80% were considered as fast recoverers/high responders. Also, subjects with recovery rates close to 40% were considered as poor responders and subjects with rates close to 80% were considered as high responders.

3.3 DNA extraction

Genomic DNA from all subjects was isolated using the two protocols described below. The method utilized depended on whether the subjects donated buccal cells or whole blood or blood spots on Whatman filter paper.

3.3.1 *Protocol 1: DNA extraction from buccal cells*

BuccalAmp QuickExtract DNA Extraction Kit (Epicentre, Australia) was used. An appropriate number of tubes containing QuickExtract DNA Extraction Solution 1.0 were removed from the kit, and labeled by number only. The subject's mouth was rinsed twice with water. Buccal cells were collected by rolling the Catch-All sample collection swab firmly on the inside of the cheek approximately 20 times on each side. The swab end containing buccal scrape was placed into a tube containing QuickExtract DNA extraction solution and rotated 5 times. The tube was then vortexed for 10 seconds and incubated at 65 ^{0}C for 1 minute. The tube was vortexed for 15 seconds and heated at 98 ^{0}C for 2 minutes. Again the tube was vortexed for 15 seconds. The DNA yield was quantified and assessed for purity by absorbance at A260 and A280. The DNA was then aliquoted and stored at -20 ^{0}C, or at -80 ^{0}C.

3.3.2 *Protocol 2: DNA extraction from blood*

GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, U.S.A.) was used to isolate DNA from whole blood. 500 μl-1 ml of blood from a finger prick was collected in a sterile microcentrifuge tube. Immediately, 100 μl of whole blood or up to a 5 μl of nucleated blood was added to 500 μl of extraction solution in a 1.5 ml centrifuge tube and

immediately mixed by vortexing. The extraction mixture was incubated for 5 min at room temperature. The entire extraction mixture was transferred to the GFX extraction column and centrifuged at 5 000 x g in a microcentrifuge (approximately 8 000 rpm) for 1 min. The flow-through was discarded. 500 µl of extraction solution was added to the column and centrifuged at 5 000 x g (approximately 8 000 rpm) for 1 min. Again the flow-through was discarded. 500 µl of wash solution was added to the column and centrifuged in a microcentrifuge at full speed (approximately 12 000-16 000 rpm) for 3 min. The collection tube was discarded and the column was transferred to a fresh 1.5 ml microcentrifuge tube (i.e. not a collection tube). 50-100 µl of pre-heated (70 ^{0}C) elution buffer or autoclaved double-distilled water was applied directly to the glass fibre matrix in the GFX column and allowed to incubate for 1 min. The purified DNA was obtained after centrifugation of the column at 5 000 x g for 1 min. The DNA samples were quantified by spectrophotometry, then aliquoted and stored at -20 ^{0}C, or at -80 ^{0}C.

3.3.3 *Protocol 3: DNA extraction from blood spots*

A blood spot was collected on a 3 cm piece of Whatman filter paper. The blood spot filter paper was cut into quarters and placed in a 1.5 ml microtube. 250 µl of 0.1% Triton X-100 was added to the tube. 15 µl of 20 mg/ml proteinase K was added and the tube was vortexed for 1 min. The tube was incubated at 50 ^{0}C for 30 mins. Again the tube was vortexed for 1 min and incubated at 50 ^{0}C for a further 30 min. 25 µl of 10X SET Buffer (500 mM Tris pH 8, 50 mM EDTA, 5% SDS) was added and the tube was mixed by vortexing. 500 of µl 1:1 chloroform/phenol (buffered with Tris pH 7.4) was added and mixed by inversion for 1 min. The tube was spun for 10 mins at 13000 rpm. Supernatant

was removed to a fresh tube and 25 μl of 3M Na acetate was added and mixed by vortexing. Isopropyl alcohol was added and the tube was vortexed, then left at -70 ^{0}C for 30 mins (or -20 ^{0}C overnight). The tube was spun for 10 mins at room temperature at 13000 rpm. Liquid was poured off gently by inverting once only. The pellet was washed once with 500 μl 70% ethanol by inverting the tube twice. The tube was then spun for 10 mins at 13000 rpm. Thereafter the tube was inverted on tissue and the pellet was left to air dry for 1 hour. The pellet was resuspended in 50 μl of water and allowed to stand at room temperature for 15 minutes. The concentration of DNA in the samples was checked by spectrophotometry as described above. Samples were aliquoted and stored at -20 ^{0}C, or at -80 ^{0}C.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 *PCR reaction to amplify the* PAX7 *polymorphic promoter region*

The DNA from all subjects was included in a PCR reaction designed to amplify the promoter region containing the variable number of tandem repeats (VNTR) in the *PAX7* gene. The promoter region was amplified with primers that span the region from -57 bp to -226 bp (GenBank accession number: AJ130875) of the *PAX7* gene and produce a product that varies in length between 163 and 172bp (Table 3.1; Figure 3.1). The PCR reaction contained a mixture of 200 μM each of dATP, dCTP, dGTP and dTTP; 1x Qiagen PCR Buffer (15 mM $MgCl_2$); 1x Q-solution (PCR additive for amplification of templates that are GC rich or that have extensive secondary structure); 0.5 units of Taq

DNA polymerase (Qiagen); 100 ng each of the forward and reverse primers and approximately 100 ng of target DNA in a final volume of 25 μl. The PCR was performed in a programmable thermal cycler (MJ Research, INC., PTC-100; U.S.A.) and cycle conditions were as follows: 94 ^{0}C, 3 minutes for 1 cycle; 94 ^{0}C, 1 minute; 56 ^{0}C, 45 seconds; 72 ^{0}C, 45 seconds for 40 cycles, followed by a final cycle of 72 ^{0}C, for 10 minutes. The PCR products were electrophoresed on a 2% agarose gel which was stained with ethidium bromide and visualized under UV light. The sizes of the PCR products were determined by reference to the electrophoretic mobility of pUC19 DNA size marker.

VNTR	Forward Primer A	Reverse Primer B	Hex Labelled Primer A1
PAX7 promoter region	5'-CGTTATTGGTCCTCCGCTC	5'-CGCGAGTGATCAGCTGGGTA	5'-CGTTATTGGTCCTCCGCTC

Table 3.1: Forward and reverse primers used to amplify the promoter polymorphic region of *PAX7*. The forward primer A1 contains a Hex-dCTP (fluorescently labeled) 5' nucleotide.

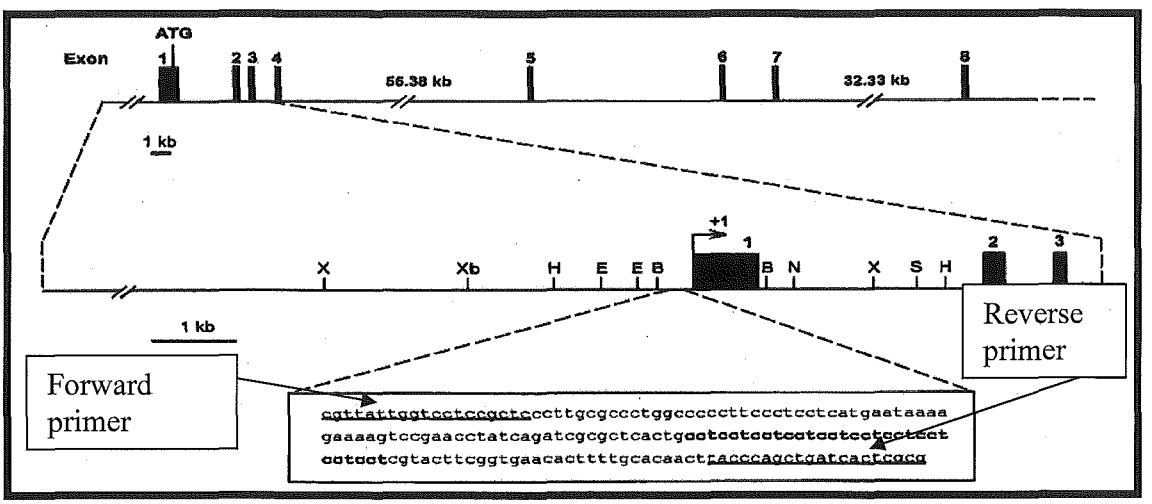
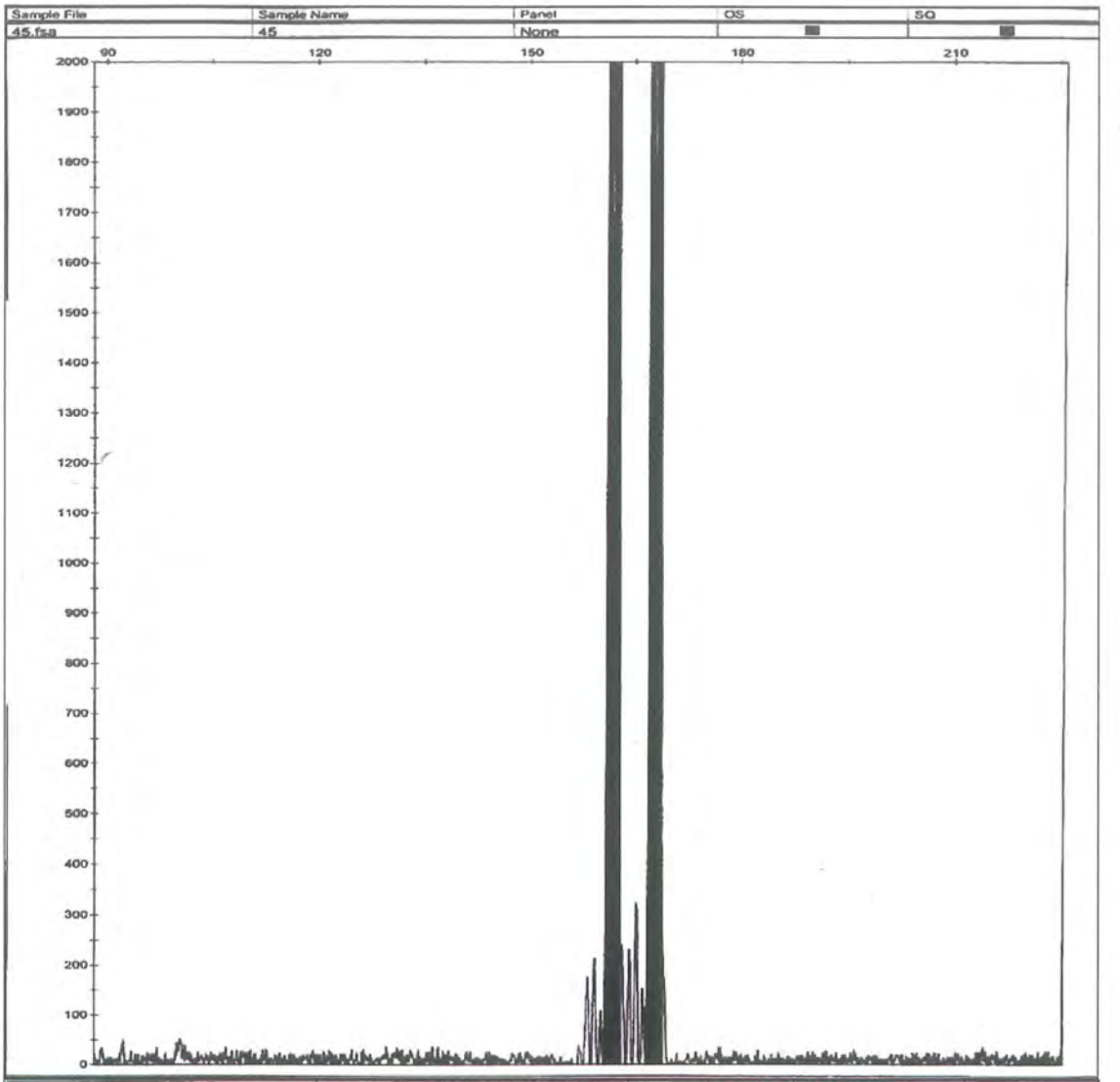


Figure 3.1: Forward and reverse primers indicated by underlined sequences, amplify a product that ranges in size from 163 to 172 bp and is positioned close to the transcription start site in the promoter region of *PAX7* (Syagailo et al., 2002).

Some of the PCR products were sequenced to confirm the correct assignment of alleles. PCR products were purified and sequenced at Royal Perth Hospital by dideoxy sequencing, using an ABI prism sequencer.

Promoter region polymorphic PCR products were further analysed by genescan at Royal Perth Hospital using a GENESCAN-3730 ABI machine as a matter of convenience to distinguish between the allelic forms which differ by only 3 bp. To do this, the PCR reaction used to amplify the polymorphic site in the promoter region of *PAX7* was performed using a fluorescently labeled forward primer A1 (Table 3.1). 5 μl of each PCR product was sent to Royal Perth Hospital for genescan analysis performed as follows: 1 μl of each PCR product was loaded on polyacrylamide gel and the gel run for less than 24 hours. A laser detector was used to detect the fluorescently labeled fragments. Information including the size and intensity of the labeled product was recorded in sample files (.fsa) format. The analysed genescan products were compared after detection by reference to a GS500LIZ_3730 size standard by the use of ABI GeneMapper® software version 3.7. The output of this software was a printout consisting of an electropherogram and a table of information, detailing bp product size and quantitative data for each peak. The peak height and area under the curve of the graphs were used to distinguish between heterozygous and homozygous individuals (Figure 3.2).



		Dye/Sample Peak	Sample File Name	Marker	Allele	Size	Height	Area	Data Point
1		G,1	45.fsa			158.4	177	1052	2789
2		G,2	45.fsa			159.47	215	1134	2803
3		G,3	45.fsa			160.38	109	425	2815
4		G,4	45.fsa			161.46	6028	36124	2829
5		G,5	45.fsa			162.45	8868	53764	2842
6		G,6	45.fsa			163.37	240	1600	2854
7		G,7	45.fsa			164.44	232	1138	2868
8		G,8	45.fsa			165.43	323	1742	2881
9		G,9	45.fsa			166.35	153	615	2893
10		G,10	45.fsa			167.42	5743	35195	2907
11		G,11	45.fsa			168.41	8359	55998	2920

Figure 3.2: An electropherogram and a table of information, detailing bp product size and quantitative data for each peak. The *PAX7* genotype for this individual was assigned as *PAX7* (8, 10).

3.4.2 *PCR reaction to amplify the* PAX7 *intron three polymorphic region*

In order to amplify the third intron of *PAX7* we initially used a set of primers (primer A & B) that amplify a region of the intron that contains two polymorphisms but this caused difficulties when analyzing PCR product sizes and therefore not pursued. To eliminate this confusion we designed a new reverse primer (primer C) that amplifies only the region containing GAAG repeats from + 4322 to + 4739 bp (GenBank accession number: NC_000001), producing a PCR product that ranges in size from 402 bp to 450 bp (Table 3.2, Figure 3.3). The PCR reaction used to amplify the intronic region of *PAX7* contained a mixture of 200 μM each of dATP, dCTP, dGTP and dTTP; 1x Qiagen PCR Buffer (15 mM $MgCl_2$); 1x Q-solution; 0.5 units of Taq DNA polymerase (Qiagen); 100 ng each of the forward and reverse primers and approximately 100 ng of target DNA in a final volume of 25 μl. The PCR was performed in a programmable thermal cycler (MJ Research, INC., PTC-100; U.S.A.) and cycle conditions were as follows: 94 ^{0}C, 5 minutes for 1 cycle; 94 ^{0}C, 1 minute; 52 ^{0}C, 1.5 minutes; 72 ^{0}C, 1 minute for 45 cycles, followed by a final cycle of 72 ^{0}C, for 10 minutes. The PCR products were electrophoresed on 3% agarose gels which were stained with ethidium bromide and visualized under UV light. The sizes of the PCR products were determined by reference to the electrophoretic mobility of a DNA size marker 1Kb plus DNA ladder.

VNTR	Forward Primer A	Reverse Primer B	Reverse Primer C
PAX7 intronic region	5'-GGAGATGATGGCTGACTG	5'-GAGTCTCTCCAGCTCCTGC	5'-TGGCTGGTGTTGCGCACAG

Table 3.2: Primer sets used to amplify the VNTR region in the third intron of *PAX7*.

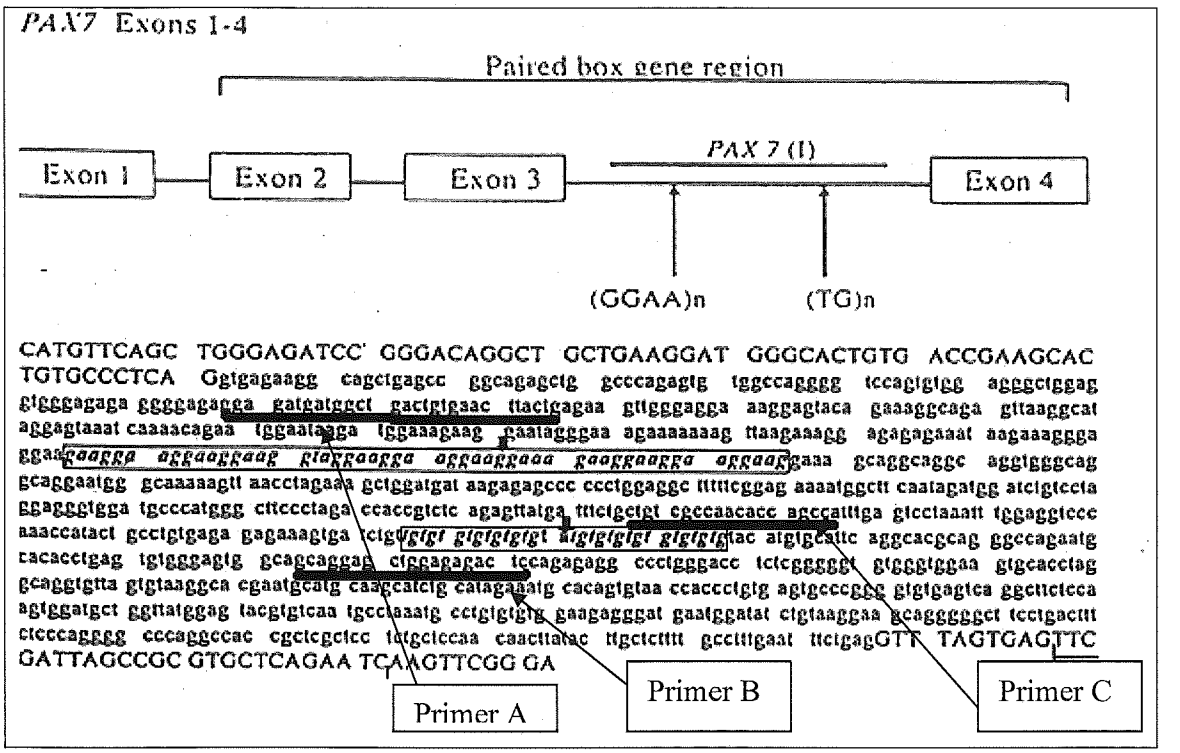


Figure 3.3: Intron primers A & B indicated by underlined sequences, amplify a 569-681 bp product containing two polymorphic repeats GAAG and TG (Ziman, Pini, Mastaglia & Kay, 2000). Intron primers A & C amplify the region containing the GAAG repeat only, producing products that range in size from 402-450 bp.

Several PCR products were sequenced to confirm the correct assignment of allelic forms. PCR products were purified and sequenced at Royal Perth Hospital by dideoxy sequencing, using an ABI prism sequencer.

3.4.3 *Statistical analysis of results*

To confirm that the study population and distribution of alleles were random and in Hardy Weinberg equilibrium, i.e. to test the validity of each sample population, we assessed the observed allelic distribution of *PAX7* relative to their expected distribution. In addition to random study population groups, we assessed the distribution of alleles in

11 Australian (Caucasian) subjects and 20 Japanese subjects exhibiting a high or a low regenerative response to eccentric exercise. Allele variation was analyzed with respect to both polymorphisms by a population genetics software package GENEPOP, available at http://wbiomed.curtin.edu.au/genepop. A Fischer's exact test was used to assess any differences in the distribution of alleles from the expected.

3.4.4 *Real Time PCR*

To classify individuals with respect to the promoter polymorphism by Real Time PCR, a Bio-Rad iQ™5 Multicolour Real-Time PCR Detection System (Biological Radiation, U.S.A.) was used (PCR amplification and fluorescence melting curve analysis). Genomic DNA (10-80 ng) was amplified using 100 ng of each forward and reverse primer; commercially available 2x SYBR Green IQ Super mix (Bio-Rad, USA) containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 u/ml *iTaq* DNA polymerase (Bio-Rad, USA), 6 mM $MgCl_2$ and stabilizers were included in a PCR reaction in a final volume of 25 μl. The Real Time conditions were as follows:

Cycle 1: (1X)		
Step 1:	95.0 °C	for 00:30.
Cycle 2: (2X)		
Step 1:	95.0 °C	for 01:00.
Cycle 3: (1X)		
Step 1:	95.0 °C	for 03:00.
Cycle 4: (45X)		
Step 1:	95.0 °C	for 00:30.
Step 2:	50.1 °C	for 00:30.
Data collection and real-time analysis enabled.		
Step 3:	72.0 °C	for 00:30.
Cycle 5: (1X)		
Step 1:	95.0 °C	for 01:00.

Cycle 6: (1X)		
Step 1:	55.0 °C	for 01:00.
Cycle 7: (397X)		
Step 1:	55.0 °C-94.6 °C	for 00:30.

The PCR was performed in standard 96-well plates, sealed with optical adhesive covers. SYBR Green binds to the minor groove of dsDNA, producing up to a 1000-fold increase in fluorescence. When the DNA denatures and becomes single stranded, there is a drop in SYBR Green fluorescence. Products were designed to be between 163 and 172 bp in length for optimal SYBR Green fluorescence melting curve analysis. After PCR amplification, the fluorescence intensity of the amplicon was measured as the temperature increased from 55 °C to 94.6 °C and measurements were taken at increments of 0.1 °C. The Real Time PCR software automatically calculated the negative derivative of the change in fluorescence and when graphed, yielded a peak at the T_m of the PCR product (Figure 3.4). Different melting temperatures were used to distinguish the three alleles.

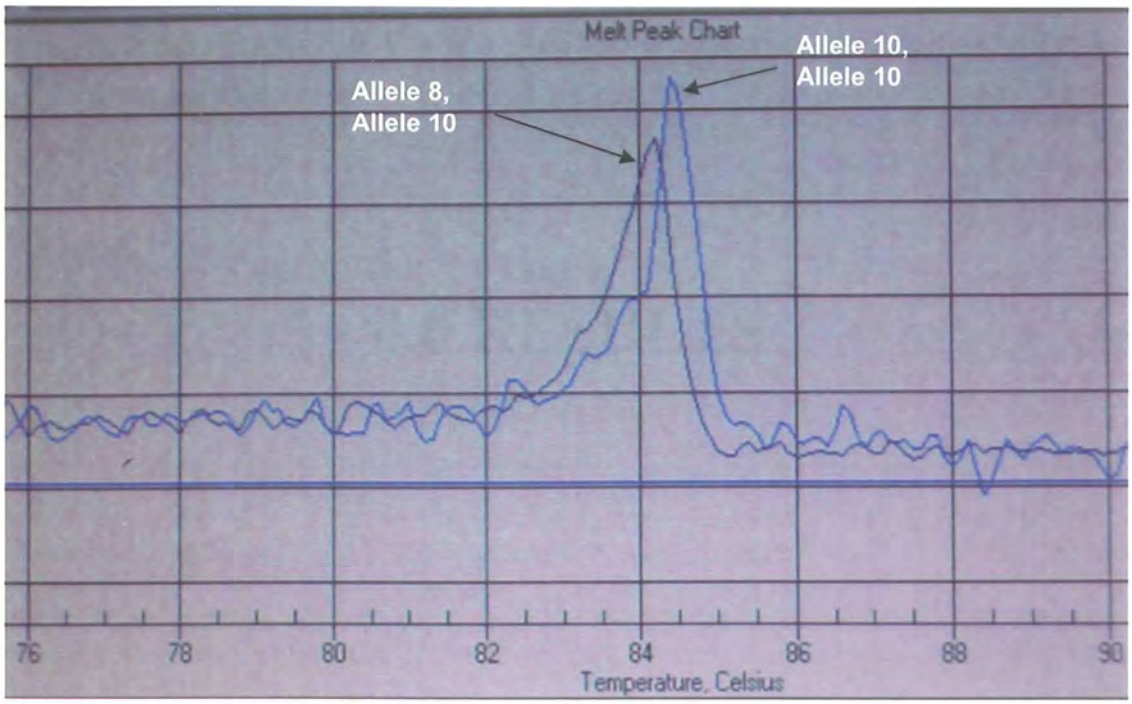


Figure 3.4. T_m curves of a homozygote (10, 10) and a heterozygote (8, 10)

4.0 RESULTS

4.1 Allele frequency in different population groups

In order to determine an association between *PAX7* allelic forms and the ability to regenerate skeletal muscle, it was necessary to assess the allele frequency of *PAX7* in the population groups to be used for the study. The variation in allele frequency in different population groups was measured with respect to polymorphisms in the promoter and intron three of *PAX7*. Whereas previous research identified these polymorphisms (Ziman et al., 2000; Syagailo et al., 2001), the allelic variation at both polymorphic sites in individuals from a variety of population groups was not measured. The results presented here indicate that the population groups utilised for the study, Australian (Caucasian), Japanese, Chinese (Han) and African (Xhosa), were in Hardy-Weinberg equilibrium (*P*-values were greater that 0.05 for differences between observed and expected allele frequencies). From our results it appears that the polymorphic sites of *PAX7*, the promoter (CCT) repeat region and the intron three (GAAG) repeat region, may be independently polymorphic. Interestingly, our results also indicate that there is a significant variation in allele frequency in different population groups when assessed at both polymorphic sites.

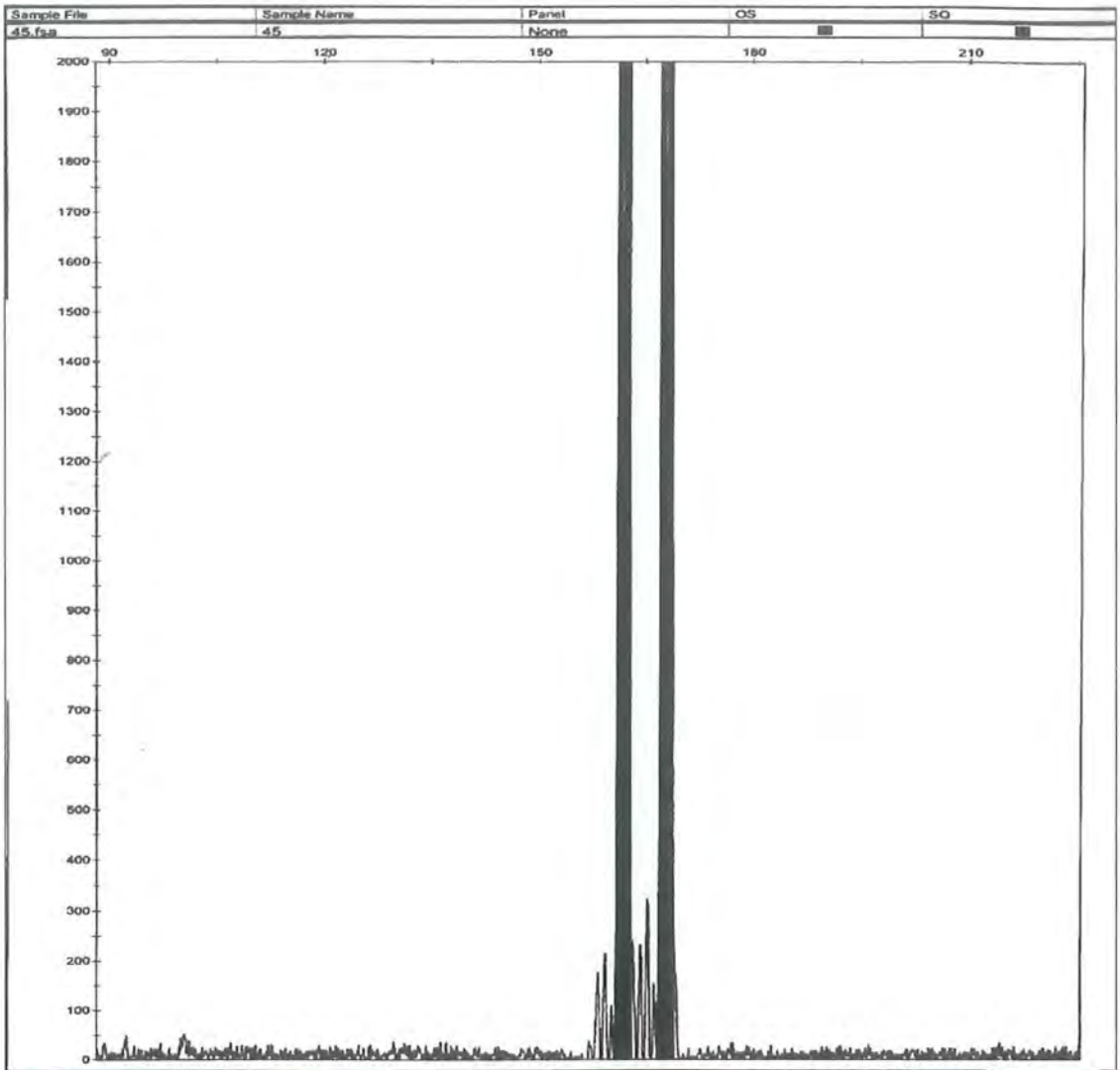
4.1.1 *Promoter polymorphism in different population groups*

In this project we measured the allele frequency with respect to the *PAX7* promoter region in Japanese, Australian (Caucasian) and Chinese (Han) population groups. Alleles were assigned by the number of trinucleotide (CCT)n repeats (Syagailo et al., 2001) in the PCR product amplified from the promoter region. PCR product size was assessed by gel electrophoresis with reference to a DNA standard (Figure 4.1). Using the primers

indicated in the methods (Figure 3.1), PCR products of the expected size of approximately 163-172 bp were obtained (Figure 4.1). However, the size difference between alleles was only 3 bp, making accurate allele assignments very difficult by standard agarose gel electrophoresis. PCR amplification of the promoter region was therefore performed with a HEX labeled forward primer A1 and the amplified product analysed by genescan.

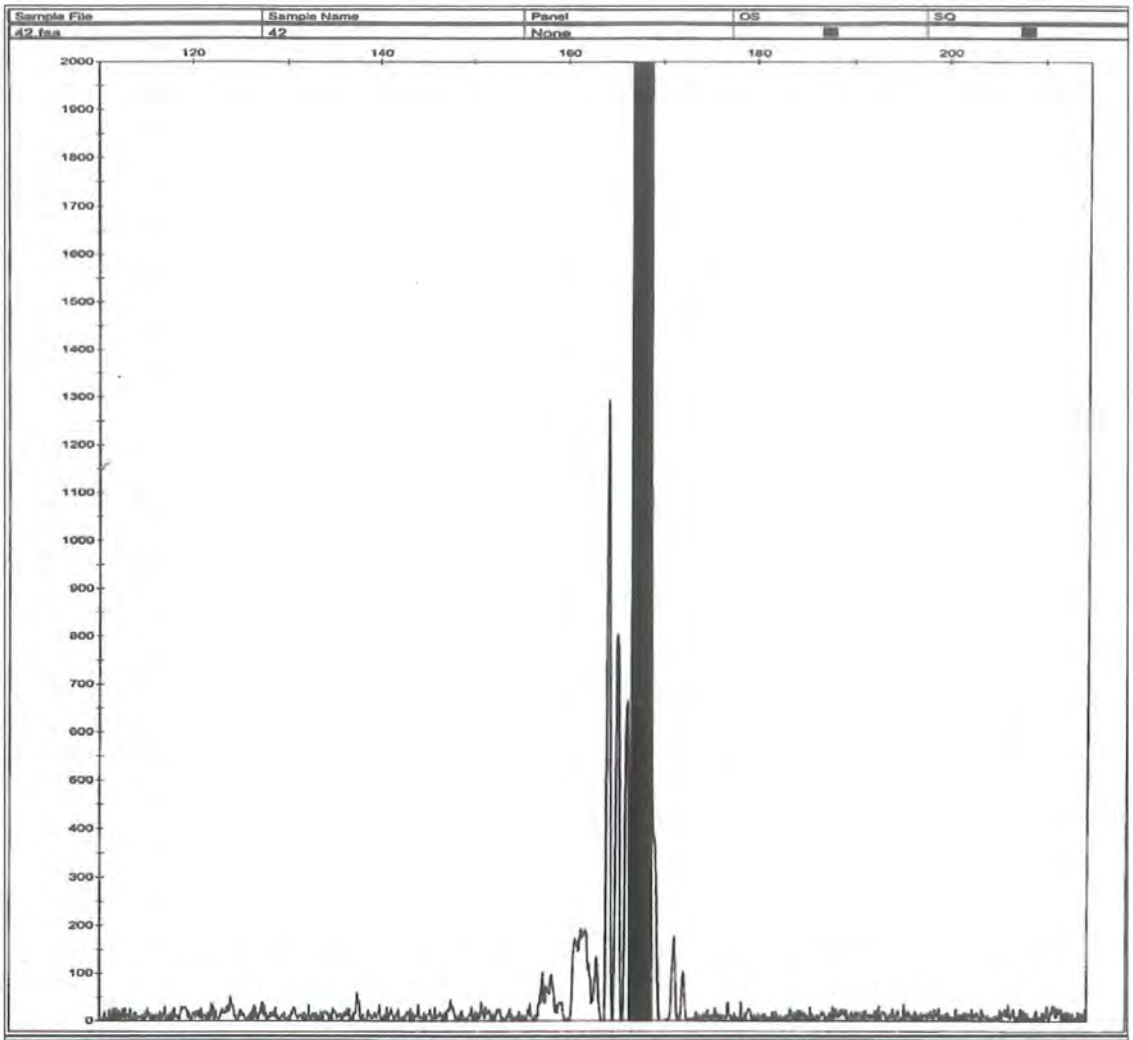
For genescan analysis, PCR products were subjected to polyacrylamide gel electrophoresis and then further analysed after detection by reference to a GS500LIZ_3730 size standard using ABI GeneMapper® software version 3.7 (Figure 4.2, Figure 4.3). Individual PCR products were also sequenced to confirm allelic assignments. Three different allelic forms were evident in all three population groups; allele 8 (8 CCT repeats), allele 10 (10 CCT repeats) and allele 11 (11 CCT repeats) confirming previous assignments (Syagailo et al., 2001).

Figure 4.1: An agarose gel illustrating PCR products obtained by amplification of the promoter region of *PAX7*. Subject DNA was from the Australian population group. The PCR product sizes vary between 163-169 bp depending on the $(CCT)_n$ repeat. The *PAX7* genotype of individual 1 was assigned *PAX7* (10, 10), lane 4; individual 2 was assigned *PAX7* (8, 10), lane 5; individual 3 was assigned *PAX7* (8, 10), lane 6; individual 4 was assigned *PAX7* (8, 10), lane 7; individual 5 was assigned *PAX7* (8, 8), lane 8; individual 6 was assigned *PAX7* (8, 10), lane 9 and individual 7 was assigned *PAX7* (8, 10), lane 10. A pUC 19 DNA ladder was used as a size marker. The genotype of all these individuals was assigned by genescan as definitive identification by standard agarose gel electrophoresis is not possible, as shown in the above figure.



		Dye/Sample Peak	Sample File Name	Marker	Allele	Size	Height	Area	Data Point
1		G,1	45.fsa			158.4	177	1052	2789
2		G,2	45.fsa			159.47	215	1134	2803
3		G,3	45.fsa			160.38	109	425	2815
4		G,4	45.fsa			161.46	6028	36624	2829
5		G,5	45.fsa			162.45	8868	58764	2842
6		G,6	45.fsa			163.37	240	1600	2854
7		G,7	45.fsa			164.44	232	1138	2868
8		G,8	45.fsa			165.43	323	1742	2881
9		G,9	45.fsa			166.35	153	615	2893
10		G,10	45.fsa			167.42	5743	35195	2907
11		G,11	45.fsa			168.41	8359	55998	2920

Figure 4.2: Genescan analysis showing distinct differences in size of PCR products obtained by amplification of the *PAX7* promoter region, when DNA from a heterozygote individual was used. The genotype for this individual was allocated *PAX7* (8, 10) by reference to the lengths of the PCR products.



		Dye/Sample Peak	Sample File Name	Marker	Allele	Size	Height	Area	Data Point
1	■	G,1	42.fsa			157.44	103	702	2682
2	■	G,2	42.fsa			158.29	98	902	2693
3	■	G,3	42.fsa			160.86	174	1404	2726
4	■	G,4	42.fsa			161.48	194	3050	2734
5	■	G,5	42.fsa			163.12	134	811	2755
6	■	G,6	42.fsa			164.36	1293	6388	2771
7	■	G,7	42.fsa			165.37	804	4405	2784
8	■	G,8	42.fsa			166.38	666	3741	2797
9	■	G,9	42.fsa			167.31	33908	193829	2809
10	■	G,10	42.fsa			168.82	21729	141262	2822
11	■	G,11	42.fsa			171.41	178	873	2862
12	■	G,12	42.fsa			172.34	104	451	2874

Figure 4.3: Genescan analysis showing the size of the PCR product obtained by amplification of the *PAX7* promoter region using DNA from a homozygote individual. The genotype for this individual was assigned as *PAX7* (10, 10) by reference to the PCR product length.

For statistical analysis of allele variation with respect to the promoter polymorphism, we used a population genetics software package GENEPOP, available at http://wbiomed.curtin.edu.au/genepop. Table 4.1 shows that none of the observed genotype frequencies in the three population groups vary significantly from the expected and all population groups are therefore in Hardy Weinberg equilibrium (P-value > 0.05). For example, in the Australian population group, genotype 1, 1 has an expected occurrence of 5.886 and the observed occurrence is 5, an insignificant difference.

Observed genotype frequencies, with respect to the promoter polymorphism for the three study population groups, are presented in Tables 4.1 and 4.2, and represented graphically in Figure 4.4. While there are significant differences between population groups, some similarities in allelic frequency are evident. Allele 10 is the most common allele in all the population groups; the frequency of this allele is consistently significantly higher than all other alleles. Specifically, in the Japanese, Chinese and Australian population groups, allele 10 has a frequency of 93%, 90.63% and 57.5% respectively. On the other hand, allele 11 is the least common allele in all population groups. In the Australian population group, allele 11 has the lowest frequency at 3.75% in comparison to allele 8 and 10 that have frequencies of 38.75% and 57.5% respectively. Similarly, allele 11 has frequencies of 1% and 1.56% in the Japanese and Chinese population groups respectively.

A.

AUSTRALIAN		
Genotypes	Number observed	Number expected
1, 1	5	5.886
2, 1	20	18.051
2, 2	12	13.101
3, 1	1	1.177
3, 2	2	1.747
3, 3	0	0.038

CHINESE		
Genotypes	Number observed	Number expected
1 , 1	0	0.159
2 , 1	4	4.603
2 , 2	27	26.238
3 , 1	1	0.079
3 , 2	0	0.921
3 , 3	0	0

JAPANESE		
Genotypes	Number observed	Number expected
1 , 1	0	0.152
2 , 1	6	5.636
2 , 2	43	43.212
3 , 1	0	0.061
3 , 2	1	0.939
3 , 3	0	0

B.

Population	*P*-value
Australian	0.8895
Chinese	0.0817
Japanese	1

Table 4.1: A Expected and observed numbers of all possible genotypes in Australian, Chinese and Japanese study population groups. **B** For the three study population groups, a Fisher's exact test was used to compare the observed and expected values and provide a *P*-value of statistical significance. *P*-values (*P*-value > 0.05) indicate no significant differences between observed and expected frequencies indicating that all the population groups are in Hardy Weinberg equilibrium.

POPULATION	Allele 8	Allele 10	Allele 11	Total
AUSTRALIAN				
Distribution of alleles	31	46	3	80
Percentage of alleles	38.75	57.5	3.75	100
JAPANESE				
Distribution of alleles	6	93	1	100
Percentage of alleles	6	93	1	100
CHINESE				
Distribution of alleles	5	58	1	64
Percentage of alleles	7.81	90.63	1.56	100

Table 4.2: Distribution of alleles and the calculated percentage of total alleles in Australian, Japanese and Chinese population groups with respect to the polymorphism in the promoter region.

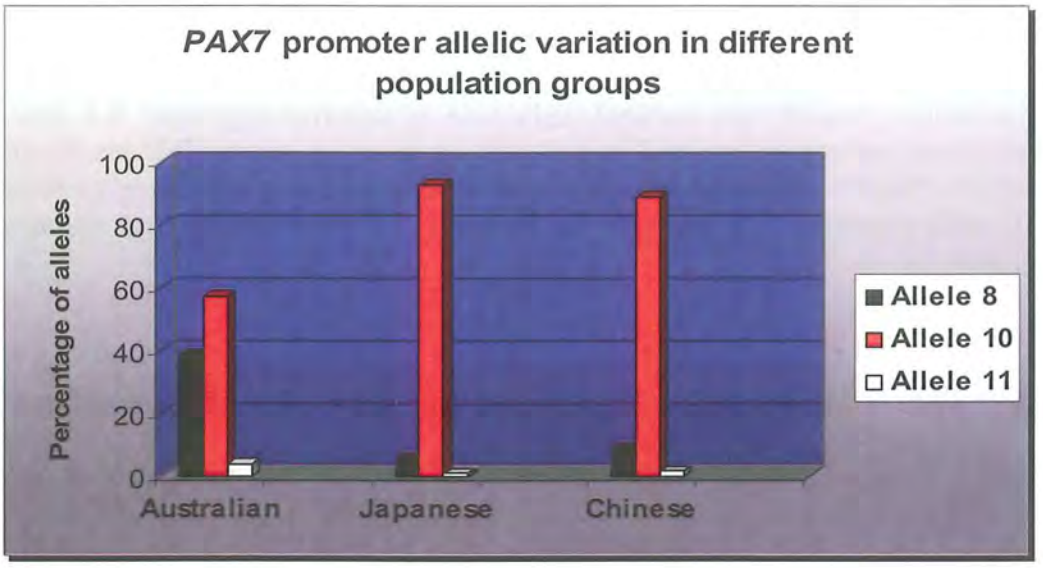


Figure 4.4: Graphical representation of the percentages of allele frequencies in Australian, Japanese and Chinese population groups.

Our results indicate that there is a statistically significant variation in allele frequencies between Australian and Asian population groups (Chinese and Japanese) (Table 4.3, Figure 4.4). For example, in the Australian population group, alleles 8, 10 and 11 have frequencies of 38.75%, 57.5% and 3.75% respectively in comparison to the Japanese population group where alleles 8, 10 and 11 have frequencies of 6%, 93% and 1% respectively. The results also indicate that alleles 8 and 11 are more common in Australians than they are in either the Japanese or Chinese.

	Genotypes:					
	1 1	1 2	2 2	1 3	2 3	
Pop:	1	2	2	3	3	All
AUS	5	20	12	1	2	40
JAP	0	6	43	0	1	50
CHI	0	4	27	1	0	32
All:	5	30	82	2	3	122

Locus	Populations	P-value	S.E.
PAX7	JAP & AUS	0.00000	0.00000
PAX7	CHI & AUS	0.00000	0.00000
PAX7	CHI & JAP	0.87819	0.00198

Table 4.3: Genotypic variation in Australian, Japanese and Chinese population groups. Significant differences in genotype frequencies between Australian and Chinese or Japanese population groups, indicated by low *P*-values (*P*-value < 0.005). In this table, genotype 1, 2 represents allele 8, allele 10 and genotype 2, 3 represents allele 10, allele 11.

From these results it is evident that there are striking differences in allele frequencies between Australian and Japanese or Chinese population groups, yet in all groups, allele 10 is the most common and allele 11 is the least common.

4.1.2 *Intron polymorphism in different population groups*

In addition to assessing allelic variation with respect to the *PAX7* promoter polymorphic region, we have also measured the allele frequency with respect to the intron three polymorphic region using Japanese, Australian (Caucasian), Chinese (Han) and African (Xhosa) population groups. Alleles were allocated by PCR amplification of the intron three region that contains the GAAG repeats, followed by gel electrophoresis and reference of product size to a DNA standard (Figure 4.5). Using the primers indicated in the methods section (Figure 3.3), PCR products between 400 and approximately 460 bp were obtained which corresponds to the expected size of between 414 bp and 434 bp (Figure 4.5). In order to confirm the allelic assignments, individual PCR products were sequenced.

Using the population groups indicated above, four different allelic forms were identified; one of these has not been reported previously. The four alleles identified were: allele 1 containing nine GAAG repeats, allele 2 containing thirteen GAAG repeats, allele 3 containing seventeen GAAG repeats and allele 4 containing twenty-one GAAG repeats (refer to Figure 3.3 for location of the $(GAAG)_n$ repeat in intron three of *PAX7*).

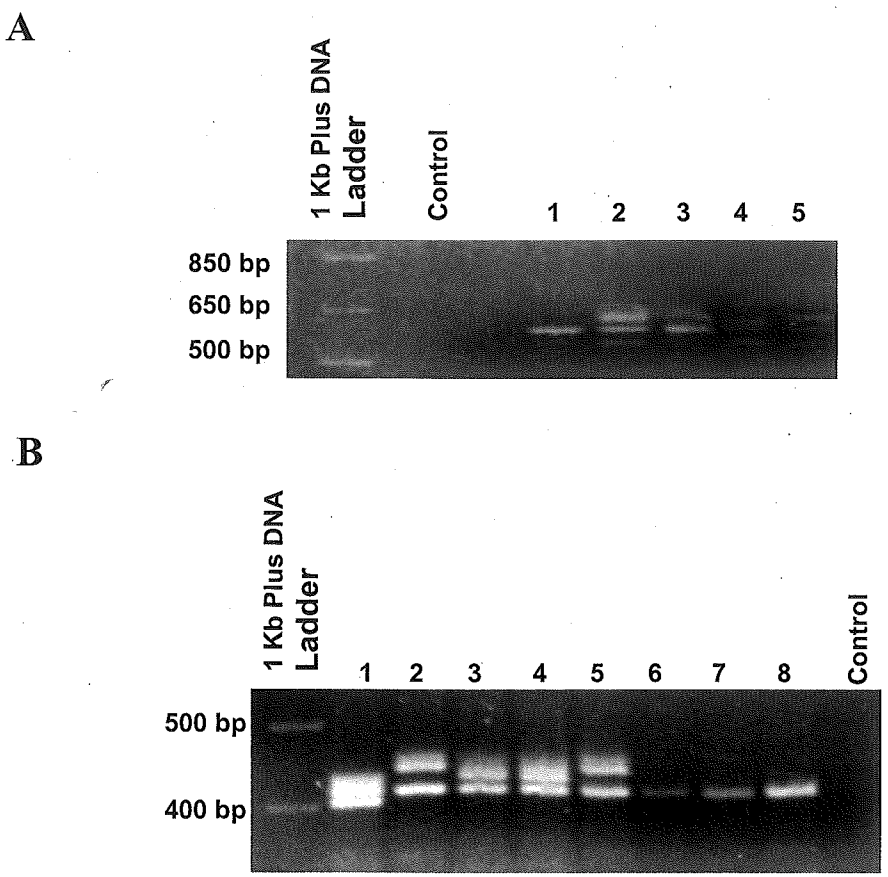


Figure 4.5: A An agarose gel showing separation of PCR products produced by amplification of the intron three region of *PAX,* using DNA isolated from subjects in the Japanese population. Oligo primers (A & B) were initially used for this amplification. The PCR products are shown for individuals 1-5. The genotype for individual 1 was allocated *PAX7* (2, 2), lane 4; individual 2 was allocated *PAX7* (2, 4), lane 5; individual 3 was allocated *PAX7* (2, 2), lane 6; individual 4 was allocated *PAX7* (2, 3), lane 7; and individual 5 was allocated *PAX7* (2, 4), lane 8. A 1 Kb Plus DNA ladder was used as a marker. Artifact bands are present in individual 2, lane 5; individual 4, lane 7 and individual 5, lane 8. **B** An agarose gel showing separation of *PAX7* alleles produced by amplification of intron three using DNA from Japanese subjects and intron primers A and C. The *PAX7* genotype in individual 1 was allocated *PAX7* (1, 2), lane 2; individual 2 was allocated *PAX7* (2, 4), lane 3; individual 3 was allocated *PAX7* (2, 3), lane 4; individual 4 was allocated *PAX7* (2, 3), lane 5; individual 5 was allocated *PAX7* (2, 4), lane 6; individual 6 was allocated *PAX7* (2, 2), lane 7, individual 7 was allocated *PAX7* (2, 2), lane 8 and individual 8 was allocated *PAX7* (2, 2), lane 9. Differences were evident using the different primer combinations (A & B and A & C) (refer to Figure 3.3). Better results were seen using new primers (A & C) making accurate allele assignments possible.

Genotype variation at the *PAX7* intron three polymorphism was statistically analyzed by the population genetics software package GENEPOP. The results reveal that none of the observed genotype frequencies in the four population groups vary significantly from the expected frequencies (Table 4.4). All four population groups were in Hardy Weinberg equilibrium with *P*-values greater than 0.05. For example, in the African population group, genotype 1, 1 has an expected occurrence of 0.017 and an observed occurrence of 0, an insignificant difference.

The observed allelic variation, with respect to the intron three polymorphism, for the four study population groups is shown in Table 4.5, Figure 4.6. Again it is evident that there are some significant similarities and differences in allele frequencies between the population groups. Our results indicate that allele 2 (thirteen GAAG repeats) is the most common in all population groups. The frequency of this allele is consistently significantly higher than that of all other alleles; in the Australian, Japanese, Chinese and African population groups, allele 2 has a frequency of 61.25%, 82.2%, 75% and 53.3% respectively. By comparison, allele 1 is the least common allele in all the population groups; in the Australian, Japanese, Chinese and African population groups, allele 1 has a frequency of 0%, 2.22%, 0% and 3.3% respectively. It is important to note that there is a consistently, statistically significant variation in the frequency of allele 3 in the population groups; distinctively, in the Japanese, Chinese, Australian, and African population groups, allele 3 has a frequency of 7.78%, 3.3%, 23.75% and 26.67% respectively. The non-occurrence of allele 1 in the Australian and Chinese population

groups may be explained by the atypical incidence of the allele and the limited sample number used in this study.

A.

AUSTRALIAN		
Genotypes	Number of observed	Number of expected
2, 2	13	14.886
3, 2	14	11.785
3, 3	1	2.165
4, 2	9	7.443
4, 3	3	2.886
4, 4	0	0.835

CHINESE		
Genotypes	Number of observed	Number of expected
2, 2	17	16.78
3, 2	1	1.525
3, 3	0	0.017
4, 2	10	9.915
4, 3	1	0.441
4, 4	1	1.322

JAPANESE		
Genotypes	Number of observed	expected
1, 1	0	0.011
2, 1	2	1.663
2, 2	29	30.348
3, 2	7	5.820
3, 1	0	0.157
3, 3	0	0.236
4, 2	7	5.820
4, 1	0	0.157
4, 3	0	0.551
4, 4	0	0.236

AFRICAN		
Genotypes	Number of observed	Number of expected
1, 1	0	0.017
2, 1	0	1.085
2, 2	6	8.407
3, 2	11	8.678
3, 1	2	0.542
3, 3	1	2.034
4, 2	9	5.424
4, 1	0	0.339
4, 3	1	2.712
4, 4	0	0.763

B.

Population	*P*-values
Australian	0.6164
Chinese	0.5805
Japanese	1
African	0.0765

Table 4.4: A Expected and observed numbers of all possible genotypes in Australian, Chinese, Japanese and African population groups. **B** For the four study population groups, a Fisher's exact test was used to compare the observed and expected values and provide a *P*-value of statistical significance. *P*-values (*P*-value > 0.05) indicate no significant differences between observed and expected frequencies in all population groups indicating that the population groups are in Hardy Weinberg equilibrium.

Population	Allele 1	Allele 2	Allele 3	Allele 4	Total
AUSTRALIAN					
Distribution of alleles	0	49	19	12	80
Allele percentage	0	61.25	23.75	15	100
CHINESE					
Distribution of alleles	0	45	2	13	60
Allele percentage	0	75	3.33	21.67	100
JAPANESE					
Distribution of alleles	2	74	7	7	90
Allele percentage	2.22	82.2	7.78	7.78	100
AFRICAN					
Distribution of alleles	2	32	16	10	60
Allele percentage	3.33	53.33	26.67	16.67	100

Table 4.5: Distribution and percentage of total alleles in Australian, Japanese, Chinese and African population groups when alleles were assigned by analysis of the polymorphism at the intron three locus.

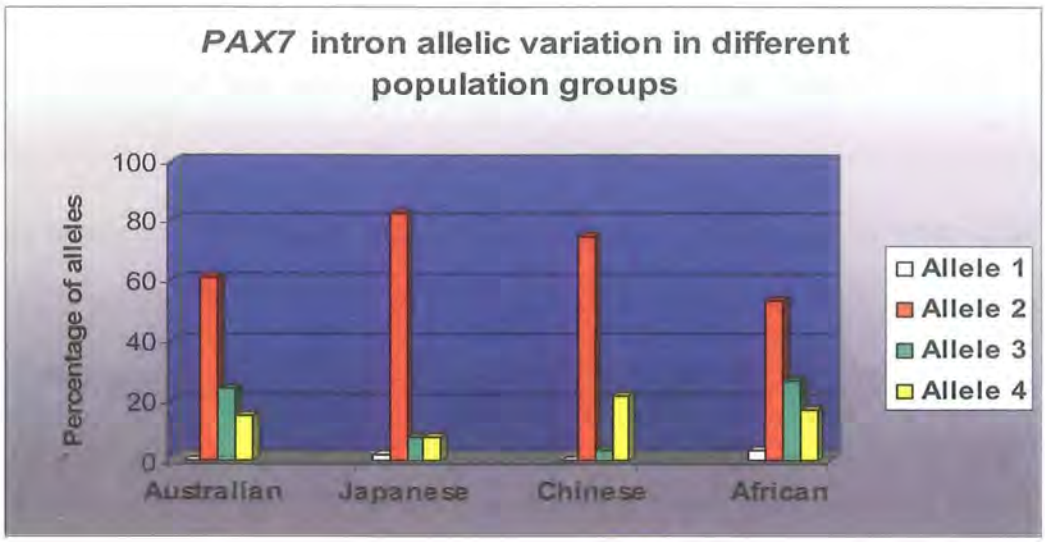


Figure 4.6: Graphical representation of observed allelic variation in the four population groups when alleles were assigned by analysis of the polymorphism at the *PAX7* intron three polymorphic locus.

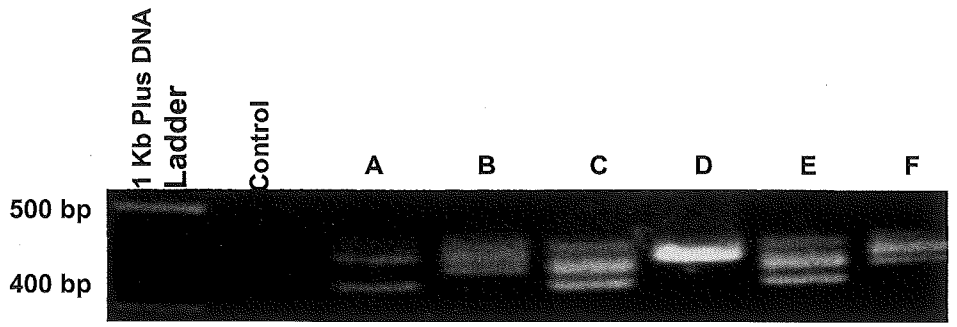
Overall, it is evident that allele 2 is the most common allele and allele 1 is the least common in all the population groups, whereas alleles 3 and 4 show remarkable differences in frequency between the population groups. Interestingly, allele frequencies of the Australian group are very similar to the African group in comparison to the Chinese and Japanese groups.

4.2 Analysis of inheritance of *PAX7* polymorphisms

To determine the heritable nature of *PAX7* alleles assigned by analysis at the two polymorphic loci, and to determine whether the loci are dependently or independently polymorphic, we analyzed the genotypes of individual members of a three generation Australian family. The genotypes of the family members were assigned and the alleles

were analyzed for coinheritance (Figure 4.7). It appears from our results that the two polymorphisms get inherited together, except for one instance. These results imply that the two *PAX7* polymorphisms, like most microsatellite markers change size over time. A larger family would need to be analysed to clarify this. Analysis of individuals from the Japanese population group do not support this idea however, since in the Japanese population group, allele 10 (promoter polymorphism) appears to be inherited with allele 2.

A



B

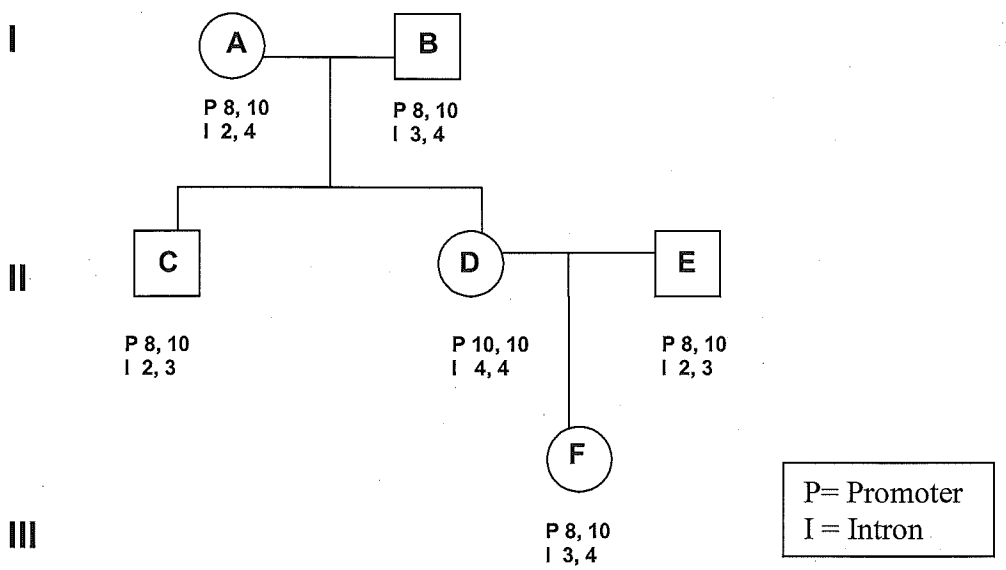


Figure 4.7: *PAX7* alleles were assigned by PCR amplification of the polymorphic region of the promoter and intron three, followed by genescan or gel electrophoresis respectively. **A** An agarose gel showing the PCR products obtained by amplification of intron three using DNA from the individual members of the Australian family. The 1Kb Plus DNA Ladder was used as a marker. **B** The family pedigree used to examine inheritance of *PAX7* alleles. Genotypes assigned for the promoter and intron polymorphic loci of each family member are indicated.

4.3 The relationship between allelic variation at the *PAX7* polymorphic loci and skeletal muscle regenerative ability

The importance of *PAX7* in muscle regeneration prompted us to investigate whether there is an association between allelic variation at the *PAX7* loci and muscle regenerative ability. The recent identification of the two polymorphic loci within *PAX7*, and our analysis of the allele frequencies at these two loci, within several population groups, made this study possible. Analysis of the promoter region containing the polymorphism (Figure 5.3) indicates that it is highly conserved, signifying suggesting functional importance. On the other hand, the region in intron three containing the polymorphism is not conserved (Figure 5.4) and the independent polymorphic nature of the two loci, suggest that allelic variation at this locus may have no functional significance. Results presented here, although preliminary in nature, support this premise.

4.3.1 *Allelic variation at the* PAX7 *promoter locus and muscle regenerative ability*

To determine an association between muscle regenerative ability and allelic variation at the *PAX7* promoter locus, we used individuals from the Australian (Caucasian) and Japanese population groups; these groups were chosen because they exhibit significantly different allele frequencies (P- value = 0.00000) (Table 4.3). For an association between muscle regenerative ability and allelic variation at the *PAX7* loci to be authentic, it would need to be evident in both population groups. Therefore we found thirty one volunteers from the two population groups, that were willing to perform the eccentric exercise

component of the study. It was necessary to choose untrained individuals as training reduces the regenerative response, making distinction between high and low regenerative response difficult to measure. A recovery rate (%) was calculated as a measure of muscle strength and therefore of regenerative ability; decreased muscle strength indicated presence of muscle damage where increased muscle strength indicated low muscle damage. We used this measure [as a marker of muscle damage and regenerative ability (Nosaka & Newton, 2002)] to show association with allelic variation in *PAX7*.

Firstly, it was necessary to show that the exercised Australian and Japanese population groups did not vary in allele frequency from those of the normal random population. Genotypes for exercised Australian and Japanese subjects were assessed with respect to the promoter *PAX7* polymorphism, and compared with those of the random population. Statistical analysis was performed using the population genetics software, GENEPOP. Results confirm similar genotype frequencies between the exercised and non-exercised subjects when assessed at the promoter polymorphic locus (Australian-*P*-value = 0.18600; Japanese- *P*-value = 0.81975) (Table 4.6 and Table 4.7).

	Genotypes:					
	1 1	2	1	2		
Pop:	1 2	2	3	3	All	
AUSE	2	0	8	0	1	11
AUS	5	20	12	1	2	40
All:	7	20	20	1	3	51

Locus	Populations	P-value	S.E.
PAX7	AUS & AUSE	0.18600	0.00502

Table 4.6: Genotype frequencies at the *PAX7* promoter locus in normal random Australian (AUS) and exercised Australian (AUSE) subjects. Genepop analysis reveals that the genotype frequencies in the two groups are not statistically different (*P*-value = 0.18600). In this table, genotype 1, 2 represents allele 8, allele 10 and genotype 2, 3 represents allele 10, allele 11.

	Genotypes:				
	1 2	2	3		
Pop:	2 2	3	3	All	
JAPE	3	16	0	1	20
JAP	6	43	1	0	50
All:	9	59	1	1	70

Locus	Populations	P-value	S.E.
PAX7	JAPE & JAP	0.81975	0.00263

Table 4.7: Genotype frequencies at the *PAX7* promoter locus in normal random Japanese (JAP) and exercised Japanese (JAPE) subjects. Genepop analysis reveals that the genotype frequencies in the two groups are not statistically different (P-value = 0.81975). In this table, genotype 1, 2 represents allele 8, allele 10 and genotype 2, 3 represents allele 10, allele 11.

4.3.2 *Analysis of* PAX7 *promoter locus allelic variation in Australian exercised subjects*

According to the rate of recovery of force, the exercised Australian population was sub-divided into low (1) and high (10) responders. The genotype frequencies of random Australian and exercised Australian high responder subjects appears to be different; for example, the most common genotype in the normal, random Australian population is a 1, 2 heterozygote (*PAX7* 8, 10) whereas in the Australian exercised high responders, the most common genotype is a 2, 2 homozygote (*PAX7* 10, 10) (Table 4.8). Moreover, an analysis of the allele frequencies indicates that there is a slight difference in allele frequencies between the normal, random population group and the exercised high responder group when alleles are calculated with respect to the *PAX7* promoter polymorphism (Table 4.9 and Figure 4.7). Because of the low number of subjects, differences in the genotype frequencies between the normal, random Australian population group and the exercised high responders are not statistically significant (*P*-value = 0.28516) (Table 4.8).

	Genotypes:					
	1 1	2	1	2		
Pop:	1 2	2	3	3	All	
AUSEH	2 0	7	0	1	10	
AUS	5 20	12	1	2	40	
All:	7 20	19	1	3	50	

Locus	Populations	P-value	S.E.
PAX7	AUS & AUSEH	0.28516	0.00496

Table 4.8: Genotype frequencies of normal random Australian (AUS) and exercised Australian high responder (AUSEH) subjects with respect to the promoter locus. Genepop analysis indicates that the allele frequencies in the two groups are statistically different *P*-value = 0.28516. In this table, genotype 1, 2 represents allele 8, allele 10 and genotype 2, 3 represents allele 10, allele 11.

Population	Allele 8	Allele 10	Allele 11	Total
AUSTRALIAN (exercised-high responders)				
Distribution of alleles	4	15	1	20
Percentage of alleles	20	75	5	100
AUSTRALIAN (exercised-low responders)				
Distribution of alleles	0	2	0	2
Percentage of alleles	0	100	0	100
AUSTRALIAN (normal)				
Distribution of alleles	31	46	3	80
Percentage of alleles	38.75	57.5	3.75	100

Table 4.9: Distribution and percentage of *PAX7* alleles, assigned with respect to the promoter polymorphism, in random and exercised Australian high and low responder subjects.

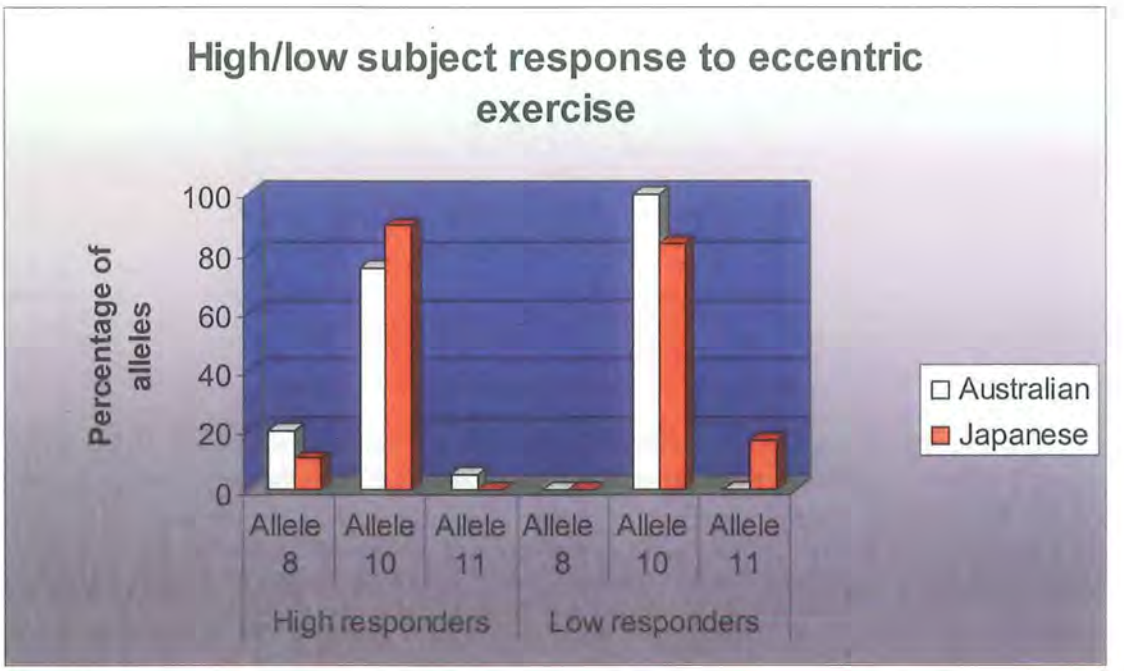


Figure 4.8: Allelic variation at the *PAX7* promoter polymorphic locus, for Australian and Japanese high and low responder subjects.

While there may be a difference between the genotypes and allele frequencies in the exercised Australian, low responder subjects relative to the normal, random Australian population group, only one Australian exercised subject was a low responder, with a *PAX7* 10, 10 (2, 2) homozygote genotype. An analysis of allele frequencies between the normal, random population group and the exercised low responder are given in Table 4.9 and depicted in Figure 4.8. Because of the low subject number, differences in the allele frequencies between the normal, random Australian population group and the exercised low responders are not statistically significant (*P*- value = 0.05) (Table 4.10) and hold no validity.

	Genotypes:					
	1	1	2	1	2	
Pop:	1	2	2	3	3	All
AUSEL	0	0	1	0	0	1
AUS	5	20	12	1	2	40
All:	5	20	13	1	2	41

Locus	Populations	P-value	S.E.
PAX7	AUSEL & AUS	0.50858	0.00701

Table 4.10: Genotype frequencies of normal random Australian (AUS) and exercised Australian low responder (AUSEL) subjects with respect to the promoter locus. Genepop analysis indicates that the allele frequencies in the two groups are not statistically different *P*-value = 0.5. However, low subject numbers do not allow accurate statistical measurements to be made. In this table, genotype 1, 2 represents allele 8, allele 10 and genotype 2, 3 represents allele 10, allele 11.

4.3.2 *Analysis of allelic variation in Japanese exercised subjects at the* PAX7 *promoter locus*

The exercised Japanese population was sub-divided into low (6) and high (14) responders according to rates of recovery of force after eccentric exercise. The genotype frequencies of exercised Japanese high responder subjects relative to the random Japanese population group appears to be unchanged at the *PAX7* promoter locus; for example, the most common genotype in the normal, random Japanese and in the Japanese exercised high responder population is a *PAX7* 10, 10 homozygote (Table 4.11). Moreover, there is no difference in allele frequencies between the normal, random population group and the exercised high responder group when alleles are calculated with respect to the *PAX7* promoter polymorphism (Table 4.12 and Figure 4.7). The similarities in the allele

frequencies between the exercised high responders and the normal, random Japanese population are confirmed by statistical analysis (*P*-value = 0.74195) (Table 4.11).

	Genotypes:			
	1	2	2	
Pop:	2	2	3	All
JAPEH	3	11	0	14
JAP	6	43	1	50
All:	9	54	1	64

Locus	Populations	P-value	S.E.
PAX7	JAPEH & JAP	0.74195	0.00292

Table 4.11: Genotype variation in random Japanese (JAP) and exercised Japanese high responder (JAPEH) subjects. Genepop analysis indicates that the allele frequencies in the two groups are not statistically significantly different. *P*-value = 0.74195. In this table, genotype 1, 2 represents allele 8, allele 10 and genotype 2, 3 represents allele 10, allele 11.

Population	Allele 8	Allele 10	Allele 11	Total
JAPANESE (exercised-high responders)				
Distribution of alleles	3	25	0	28
Percentage of alleles	10.71	89.29	0	100
JAPANESE (exercised-low responders)				
Distribution of alleles	0	10	2	12
Percentage of alleles	0	83.33	16.67	100
JAPANESE (normal)				
Distribution of alleles	6	93	1	100
Percentage of alleles	6	93	1	100

Table 4.12: Distribution and percentage of alleles with respect to the promoter polymorphism in random and exercised, Japanese high and low responder subjects.

Similarly, there is little difference in allelic variation at the *PAX7* promoter locus between exercised Japanese low responder and random Japanese subjects; for example, the most common genotype in the Japanese exercised low responders and in the normal, random Japanese population is a *PAX7* 10, 10 homozygote. Moreover, an analysis of the allele frequencies indicates that there is no significant difference in allele frequencies between the normal, random population group and the exercised low responder group (Table 4.12 and Figure 4.8). Differences in the allele frequencies between the normal, random Japanese population group and the exercised low responders are not statistically significant (*P*- value = 0.08213) (Table 4.13).

	Genotypes:				
	1	2	2	3	
Pop:	2	2	3	3	All
JAPEL	0	5	0	1	6
JAP	6	43	1	0	50
All:	6	48	1	1	56

Locus	Populations	P-value	S.E.
PAX7	JAPEL & JAP	0.08213	0.00249

Table 4.13: Genotype variation in random Japanese (JAP) and exercised Japanese low responder (JAPEL) subjects. Genepop analysis indicates that the allele frequencies in the two groups are not statistically different *P*-value = 0.08213. In this table, genotype 1, 2 represents allele 8, allele 10 and genotype 2, 3 represents allele 10, allele 11.

Although there is no obvious association of this *PAX7* polymorphism with muscle regeneration, our preliminary data (Figure 4.8 and Table 4.14), show that there may be a trend towards low responders being associated with allele 11 (11 CCT repeats), while the high responders may be associated with allele 8 (8 CCT repeats) in the Australian and Japanese population groups. The low sample numbers make it impossible to accurately assess allele association at the promoter polymorphic locus of PAX7 with skeletal muscle regenerative ability. Larger sample numbers may provide a clearer association.

Population	Allele 8	Allele 10	Allele 11	Total
AUSTRALIAN (exercised-high responders)				
Distribution of alleles	4	15	1	20
Percentage of alleles	20	75	5	100
AUSTRALIAN (exercised- low responders)				
Distribution of alleles	0	2	0	2
Percentage of alleles	0	100	0	100
JAPANESE (exercised- high responders)				
Distribution of alleles	3	25	0	28
Percentage of alleles	10.71	89.29	0	100
JAPANESE (exercised- low responders)				
Distribution of alleles	0	10	2	12
Percentage of alleles	0	83.33	16.67	100

Table 4.14: Distribution and percentage of alleles with the respect to the promoter polymorphism in exercised Australian and Japanese high and low responder subjects.

4.3.3 *Allelic variation at the* PAX7 *intron locus and muscle regenerative ability*

Australian (Caucasian) and Japanese population groups were also used to determine an association between muscle regenerative ability and allelic variation at the intron three

polymorphic locus of *PAX7*. The allelic variation at intron three was assessed in twenty six volunteers that were willing to perform the eccentric exercise component of the study.

To validate our results, it was necessary to confirm that the exercised Australian and Japanese groups did not vary in genotype frequency from the normal random population with respect to the *PAX7* intron three polymorphism. Genotype frequencies were compared using the population genetics software program, GENEPOP. Our results show that the Australian exercised group showed no difference in allele variation relative to the random population and were therefore in Hardy Weinberg equilibrium (Table 4.15). Unfortunately, there are significantly different genotype frequencies in the Japanese exercised population group relative to the random population indicating that the exercised population is not in Hardy-Weinberg equilibrium (*P*- value = 0.00894) (Table 4.16).

	Genotypes:						
	2 2	2 3	3 3	2 4	3 4	4 4	
Pop:							All
AUSE	3	1	1	0	2	0	7
AUS	13	14	1	9	3	0	40
All:	16	15	2	9	5	0	47

Locus	Populations	P-value	S.E.
PAX7	AUSE & AUS	0.69265	0.00404

Table 4.15: Genotype frequencies of normal random Australian (AUS) and exercised Australian (AUSE) subjects with respect to the intron three polymorphism. Genepop analysis reveals that the allele frequencies in the two groups are not statistically different indicating that the exercised population group is in Hardy-Weinberg equilibrium (*P*-value = 0.69265).

	Genotypes:							
	1	2	2	1	2	3	4	
Pop:	2	2	3	4	4	4	4	All
JAPE	0	8	0	2	6	2	1	19
JAP	2	29	7	0	7	0	0	45
All:	2	37	7	2	13	2	1	64

Locus	Populations	P-value	S.E.
PAX7	JAPE & JAP	0.00894	0.00110

Table 4.16: Genotype frequencies of normal random Japanese (JAP) and exercised Japanese (JAPE) subjects with respect to the polymorphism in intron three of *PAX7*. Genepop analysis reveals that the allele frequencies in the two groups are statistically different indicating that the exercised population group is not in Hardy-Weinberg equilibrium (*P*-value = 0.00894).

4.3.4 *Analysis of allelic variation at the* PAX7 *intron three locus in Australian exercised subjects*

Genotype analysis at the intron locus was only obtained in seven subjects and they were all high responders. The genotype frequencies of random Australian and exercised Australian high responder subjects with respect to the intron polymorphism, are not statistically significantly different (p=0.69846); however, the most common genotype in the normal, random Australian population is a 2, 3 heterozygote (Table 4.17) whereas in the Australian exercised high responders, the most common genotype is a 2, 2 homozygote (Table 4.17). This difference is not significant and there is no significant difference in allele frequencies between the normal, random population group and the exercised high responder group when alleles are calculated with respect to the *PAX7* intron polymorphism (Table 4.18 and Figure 4.9). It was not possible to ascertain

differences between the genotypes and allele frequencies in the normal, random Australian group and the exercised Australian, low responder subjects as there were no low responder subjects (Table 4.18).

	Genotypes:						
	2	2	3	2	3	4	
Pop:	2	3	3	4	4	4	All
AUSEH	3	1	1	0	2	4	7
AUS	13	14	1	9	3	4	40
All:	16	15	2	9	5	0	47

Locus	Populations	P-value	S.E.
PAX7	AUSEH & AUS	0.69846	0.00369

Table 4.17: Genotype frequencies of normal random Australian (AUS) and exercised Australian high responder (AUSEH) subjects with respect to the intron three polymorphism. Genepop analysis reveals that the allele frequencies in the two groups are not statistically different indicating that the exercised population group is in Hardy-Weinberg equilibrium (*P*-value = 0.69265).

Population	Allele 1	Allele 2	Allele 3	Allele 4	Total
AUSTRALIAN (exercised-high responders)					
Distribution of alleles	0	7	5	2	14
Allele percentage	0	50	35.71	14.29	100
AUSTRALIAN (exercised-low responders)					
Distribution of alleles	0	0	0	0	0
Allele percentage	0	0	0	0	0
AUSTRALIAN (normal)					
Distribution of alleles	0	49	19	12	80
Allele percentage	0	61.25	23.75	15	100

Table 4.18: Distribution and percentage of alleles with respect to the intron polymorphism in random and exercised Australian, high and low responder subjects.

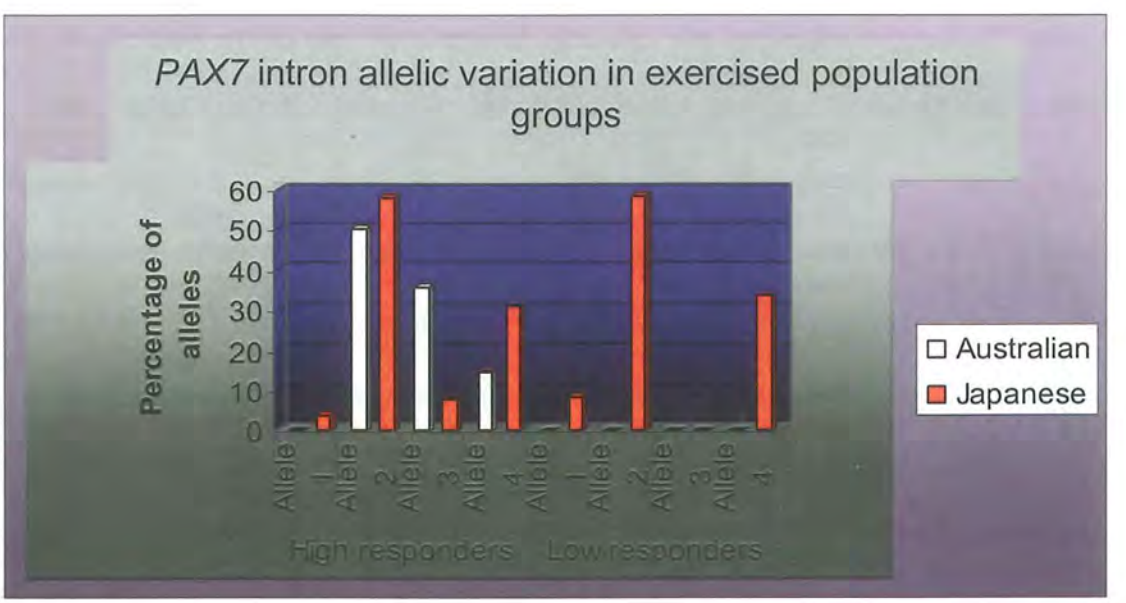


Figure 4.9: Allelic variation at the *PAX7* intron three polymorphic locus in Australian and Japanese subjects that were high or low responders to eccentric exercise.

4.3.5 *Analysis of allelic variation at the* PAX7 *intron three locus in Japanese exercised subjects*

The exercised Japanese population was arranged into high (13) and low (6) responders. The genotype frequencies in exercised Japanese high responder subjects differ from that of the random Japanese population group (*P*- value = 0.03745) (Table 4.19); while the commonest genotype in the normal, random Japanese and in the Japanese exercise high responders population is a 2, 2 homozygote (Table 4.19), the distribution of the other genotypic forms differ from those of the random Japanese population (p=0.03745, Table 4.19). Similarly an analysis of the allele frequencies indicates that there is a difference in allele frequencies between the normal, random population group and the exercised high responder group when alleles are calculated with respect to the *PAX7* intron polymorphism in the Japanese groups (Table 4.20 and Figure 4.9). There also appears to be a significant difference between the genotypes and allele frequencies in the normal, random Japanese group and the exercised Japanese, low responder subjects (*P* -value = 0.01752, Table 4.21). For example, the most common genotype in the normal, random Japanese population and in the Japanese exercise high responders population is a 2, 2 homozygote, where as in the low responder Japanese subjects, the most common genotype is a 2, 4 heterozygote (Table 4.21). The distribution of alleles in the exercised low responder Japanese subjects differs from that of the normal, random Japanese population group but is similar to that of the high responder Japanese subjects (Table 4.20). Since the Japanese exercised subject population is not in Hardy Weinberg equilibrium and differs from that of the normal, random Japanese population group (Table 4.17), it is difficult to ascertain whether there is an association between *PAX7*

alleles, assigned with respect to the intron polymorphism, and recovery of force after eccentric exercise.

	Genotypes:							
	1	2	2	1	2	3	4	
Pop:	2	2	3	4	4	4	4	All
JAPEH	0	6	0	1	3	2	1	13
JAP	2	29	7	0	7	0	0	45
All:	2	35	7	1	10	2	1	58

Locus	Populations	P-value	S.E.
PAX7	JAPEH & JAP	0.03745	0.00225

Table 4.19: Genotype frequencies of normal random Japanese (JAP) and exercised Australian high responder (JAPEH) subjects with respect to the intron polymorphism. Genepop analysis reveals that the allele frequencies in the two groups are statistically different indicating that the exercised high responders population group is not in Hardy-Weinberg equilibrium (*P*-value = 0.69265).

Population	Allele 1	Allele 2	Allele 3	Allele 4	Total
JAPANESE (exercised-high responders)					
Distribution of alleles	1	15	2	8	26
Allele percentage	3.85	57.69	7.69	30.80	100
JAPANESE (exercised-low responders)					
Distribution of alleles	1	7	0	4	12
Allele percentage	8.33	58.33	0	33.33	100
JAPANESE (normal)					
Distribution of alleles	2	74	7	7	90
Allele percentage	2.22	82.2	7.78	7.78	100

Table 4.20: Distribution and percentage of alleles with respect to the intron polymorphism in random and exercised Japanese high and low responder subjects.

	Genotypes:					
	1	2	2	1	2	
Pop:	2	2	3	4	4	All
JAPEL	0	2	0	1	3	6
JAP	2	29	7	0	7	45
All:	2	31	7	1	10	51

Locus	Populations	P-value	S.E.
PAX7	JAPEL & JAP	0.01752	0.00143

Table 4.21: Genotype frequencies of normal random Japanese (JAP) and exercised Australian low responder (JAPEL) subjects with respect to the intron polymorphism. Genepop analysis reveals that the allele frequencies in the two groups are statistically different *P*-value = 0.69265.

In summary, it is evident that allelic variation with respect to the *PAX7* intronic polymorphism cannot explain the observed differences in recovery of force following damage by eccentric exercise. It appears that there is no direct association of the *PAX7* intron polymorphism with muscle regeneration. While data depicted in Figure 4.9 and Table 4.22 suggest that there is a trend towards high responders being associated with alleles 3 and 4 in the Australian and Japanese population groups respectively, there is a similar trend towards low responders also being associated with allele 4 in the Japanese population group (no low responder Australians were found). Clearly then, there is no specific association between allelic forms of the *PAX7* intron locus and regenerative response after eccentric exercise. Again low sample numbers make absolute association difficult to quantify.

Population	Allele 1	Allele 2	Allele 3	Allele 4	Total
AUSTRALIAN (exercised-high responders)					
Distribution of alleles	0	7	5	2	14
Allele percentage	0	50	35.71	14.29	100
AUSTRALIAN (exercised-low responders)					
Distribution of alleles	0	0	0	0	0
Allele percentage	0	0	0	0	0
JAPANESE (exercised-high responders)					
Distribution of alleles	1	15	2	8	26
Allele percentage	3.85	57.69	7.69	30.80	100
JAPANESE (exercised-low responders)					
Distribution of alleles	1	7	0	4	12
Allele percentage	8.33	58.33	0	33.33	100

Table 4.22: Distribution and percentage of alleles with respect to the intron polymorphism in exercised Australian and Japanese high and low responder subjects.

4.4 Real Time PCR

The Real time PCR method of analysis was developed in order to advance quantitative analysis of the promoter region polymorphism of *PAX7*, which would substantially reduce the cost and time of the genotype analysis. Real time PCR is based on detection and quantitation of a fluorescent reporter (e.g. SYBR Green, Hex), where the detecting signal is proportional to the amount of PCR product in a reaction. Rather than using expensive specific probes, we attempted to analyse allelic forms of *PAX7* using differences in melt curves as assessed by SYBR Green fluorescence. SYBR Green is a fluorescent intercalating agent that emits a strong fluorescent signal when it binds to double stranded DNA.

To genotype individuals' allelic forms with respect to the promoter polymorphism, we used SYBR Green fluorescence melt curves, which take advantage of the fact that different PCR product lengths melt at different temperatures. After PCR product amplification, genotyping was attempted by DNA melt analysis to assess the different length PCR products present in the sample. The negative derivative of the change in fluorescence was automatically calculated by the software (IQ™5 Multicolor Real-Time PCR Detection System Software Version 1.0) and graphed. The graphs yielded peaks at a specific T_m for different length PCR products (Figure 4.10). However, the melting temperatures were not sufficiently different to allow correct assignment of different PCR product lengths for all genotypes. Future work may involve the use of allele specific displacing probes that will provide sufficient melting temperature differences.

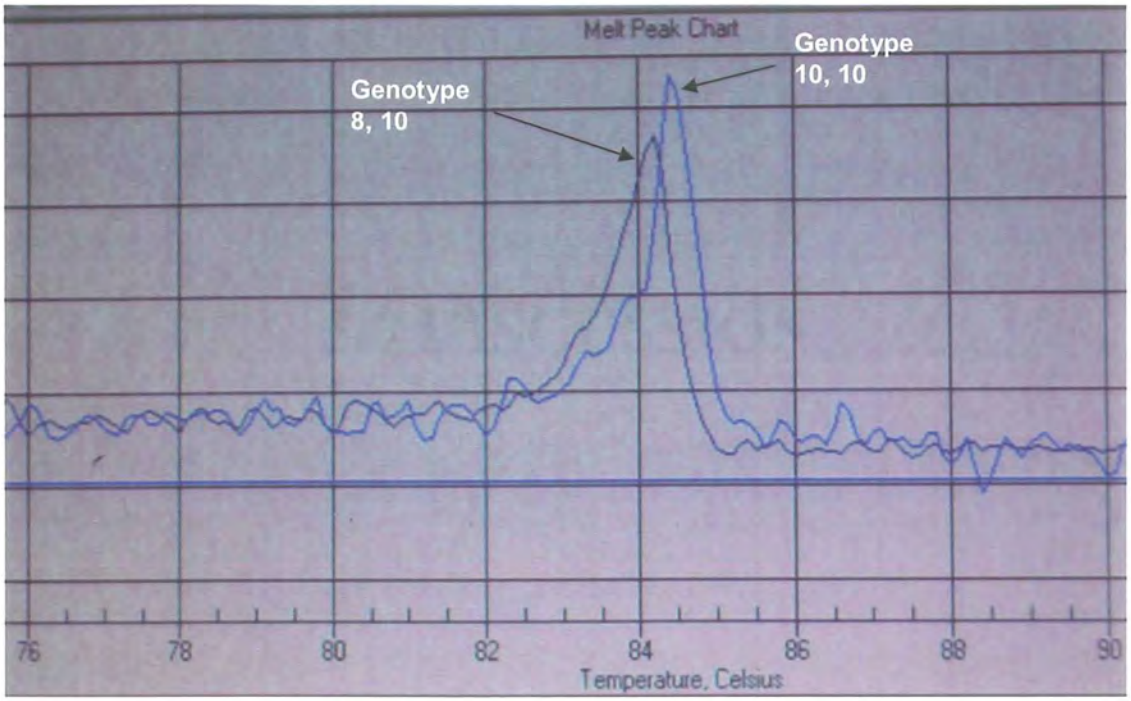


Figure 4.10: T_m curves of a homozygote (10, 10) and a heterozygote (8, 10) individuals determined by the slight change in melting temperatures.

5.0 DISCUSSION

This study determined if allelic frequencies of the gene, *PAX7*, differ in different population groups, and if promoter and intron polymorphisms are associated with observed differences in recovery of force following damage by eccentric exercise. The major findings of this study are that 1) there is a significant difference in allele frequency with respect to both *PAX7* polymorphisms in different population groups, 2) there is no strong association of the promoter polymorphism with skeletal muscle regeneration ability, but high and low responders to exercise have different allele frequencies at this polymorphic locus. This is the first study to show the allelic variation with respect to both *PAX7* polymorphisms within Australian (Caucasian), Japanese, Chinese (Han) and African (Xhosa) population groups. It is also the first study to investigate the association between allelic forms of *PAX7* and recovery of force (low/high) following eccentric exercise, indicative of muscle regenerative ability. Further studies are required to show conclusively that *PAX7* polymorphisms are associated with skeletal muscle regenerative ability.

5.1 Allele frequency in different population groups

Allele frequency in different population groups was measured with respect to polymorphisms in the promoter and intron three of *PAX7*, and revealed a large statistically significant variation in allele frequencies between Australian and Asian population groups (Chinese and Japanese), at the promoter polymorphic locus. On the other hand, analysis of *PAX7* allelic variation revealed that, at the intron locus, the Australians and Africans have a similar allele frequency distribution which differs from

that of the Japanese and Chinese population groups. While there are obvious differences between the groups, the similarities in alleles between the population groups, together with the fact that the alternate *PAX7* alleles are present in the DNA of all the subjects, indicates the presence of all alleles prior to separation of the populations and speciation.

Similar population genetic studies have shown similar differences in the distribution of several allotypes between population groups at other polymorphic loci. For example, in the *Alpha-1-Antitrypsin* gene, there is a highly significant difference in the distribution of alleles between the white and black populations of America (U.S.A.), which reflects different population group origins (De Croo, Kamboh & Ferrel, 1991). The population differentiation that is observed in these studies together with our own, indicates that differences may have arisen largely from genetic drift because of recent geographic separation (Destro-Bisol, Spedini & Pascali, 2000).

In addition we have analysed the co-inheritance of alleles assigned at the two polymorphic loci. The family study revealed that generally the two polymorphisms are inherited together, as is expected as they are located in close proximity on the chromosome (less than six kilobases apart). The difference in size of one polymorphism in one family member implies that the size of one allele has changed over time as expected for microsatellites which are unstable. Thus 5 out of 6 events in the family are dependently polymorphic. The one case where the polymorphisms are not inherited together could be due to a number of reasons. Firstly, there could have been a cross-over event between individuals B and C (unlikely). Secondly, individual B may not be the

father of individual C. Lastly, the polymorphisms may have changed size between individuals B and C. In order to more clearly analyze the co-inheritance of these polymorphisms, larger population and family studies are required.

5.2 Promoter and intron *PAX7* polymorphisms and eccentric exercise

Recent studies indicate that polymorphic variations in genes coding for muscle proteins such as *alpha-actinin 3* (*ACTN3*) and *myosin light chain kinase* (*MLCK*), contribute to the large variability in response to eccentric exercise muscle damage (Clarkson, Zambraski, Gordish-Dressman, Kearns, Hubal, Harmon & Devaney, 2005). Similarly, a polymorphism in the *cicliary neurotrophic factor receptor* (*CNTFR*) gene is associated with differences in muscle strength (Roth, Metter, Lee, Hurley & Ferrell, 2003). Such studies indicate that muscle regenerative ability is not directly related to a specific gene or group of genes, but rather shows that there are several different mechanisms responsible for the large variability in response to muscle damage. In this study we focused on the polymorphic variation in the gene *PAX7*, and its association with variability in muscle regeneration. *PAX7* is an important developmental transcription factor, therefore *PAX7* allelic variation may indeed contribute to the observed differences in recovery of force following damage by eccentric exercise. Even though there is no strong statistically significant association between promoter allelic variation and the observed differences in recovery of force following damage by eccentric exercise, a difference between the allele frequencies at the *PAX7* promoter locus, in the high and low

responders, was observed in this preliminary study. Interestingly, there is a trend toward high responders being associated with allele 8 (8 CCT repeats) and a trend towards low responders being associated with allele 11 (11 CCT repeats).

Recent studies have shown that the increased length of the repeat-polymorphism in the *Pax7* promoter increases transcriptional efficiency *in vitro* (Syagailo et al., 2002). According to our experimental data, the alleles with increased length at the promoter may decrease the transcriptional efficiency *in vivo*, or alternately increased *PAX7* expression may not be associated with better recovery of force following eccentric exercise. Several factors would be consistent with the length of the promoter polymorphism having an effect (whether increased or decreased) on transcriptional efficiency *in vivo*. Firstly, the increased length of the promoter may help the DNA molecule to unwind. The increased length of the polymorphic promoter region may have an affect on the single stranded DNA folding as indicated in Figure 5.1. This may affect the rate of transcription as the polymorphic region is upstream of the mRNA transcript start site (Gilbert, 2000, p. 119). The increased length may also assist or inhibit protein complex binding and affect the start of transcription; the transcription factor proteins predicted to bind to the promoter (Figure 5.2) may bind more efficiently to their targets when separated by increased lengths, resulting in enhanced transcription. Alternatively, an increased length may lessen protein-protein interactions between these transcription factors and reduce the rate of transcription. More specifically, increased promoter length may be advantageous for nearby activators such as AP2 and NF1 (Figure 5.2) (Syagailo et al., 2002, & Gilbert, 2000, p. 175) and allow them to bind more efficiently to specific enhancer sequences and

interact with coactivators bound to enhancer-silencer sequences in the *PAX7* promoter. Alternatively, the increased length may reduce the interaction between AP2 and NF1 and reduce *PAX7* transcription *in vivo*.

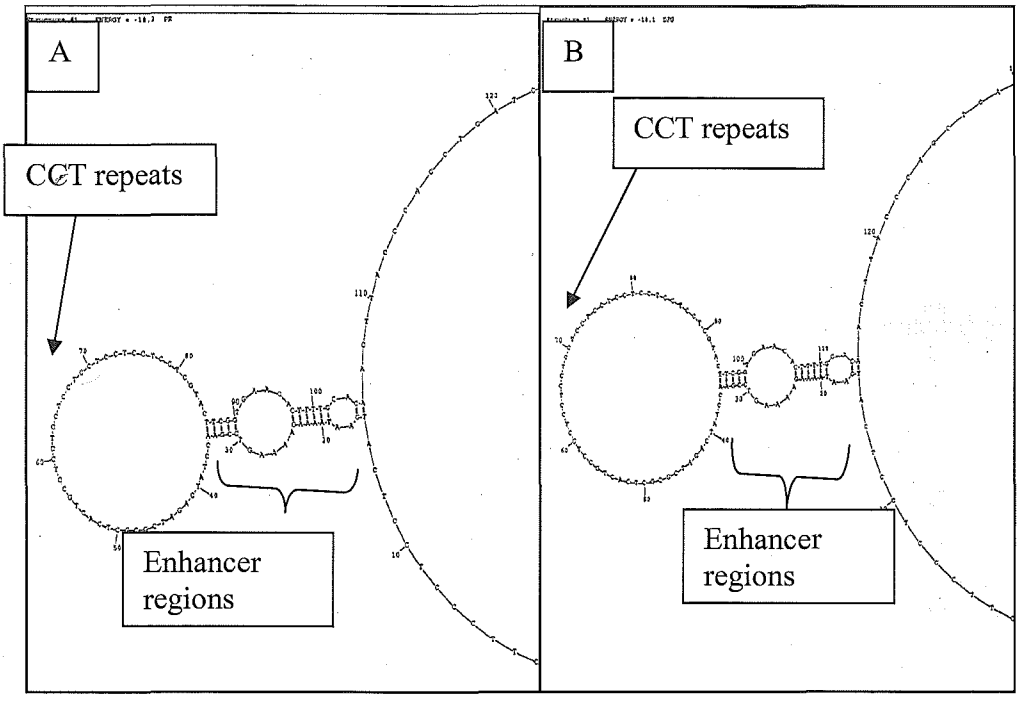


Figure 5.1: DNA folding of the *PAX7* promoter region containing the polymorphism. **A** DNA folding containing allele 8 (8 CCT repeats). **B** DNA folding containing allele 11 (11 CCT repeats). Increased CCT repeat number appears to have an affect on DNA folding by opening the molecule further, possibly affecting binding of transcription factors in nearby enhancer regions.

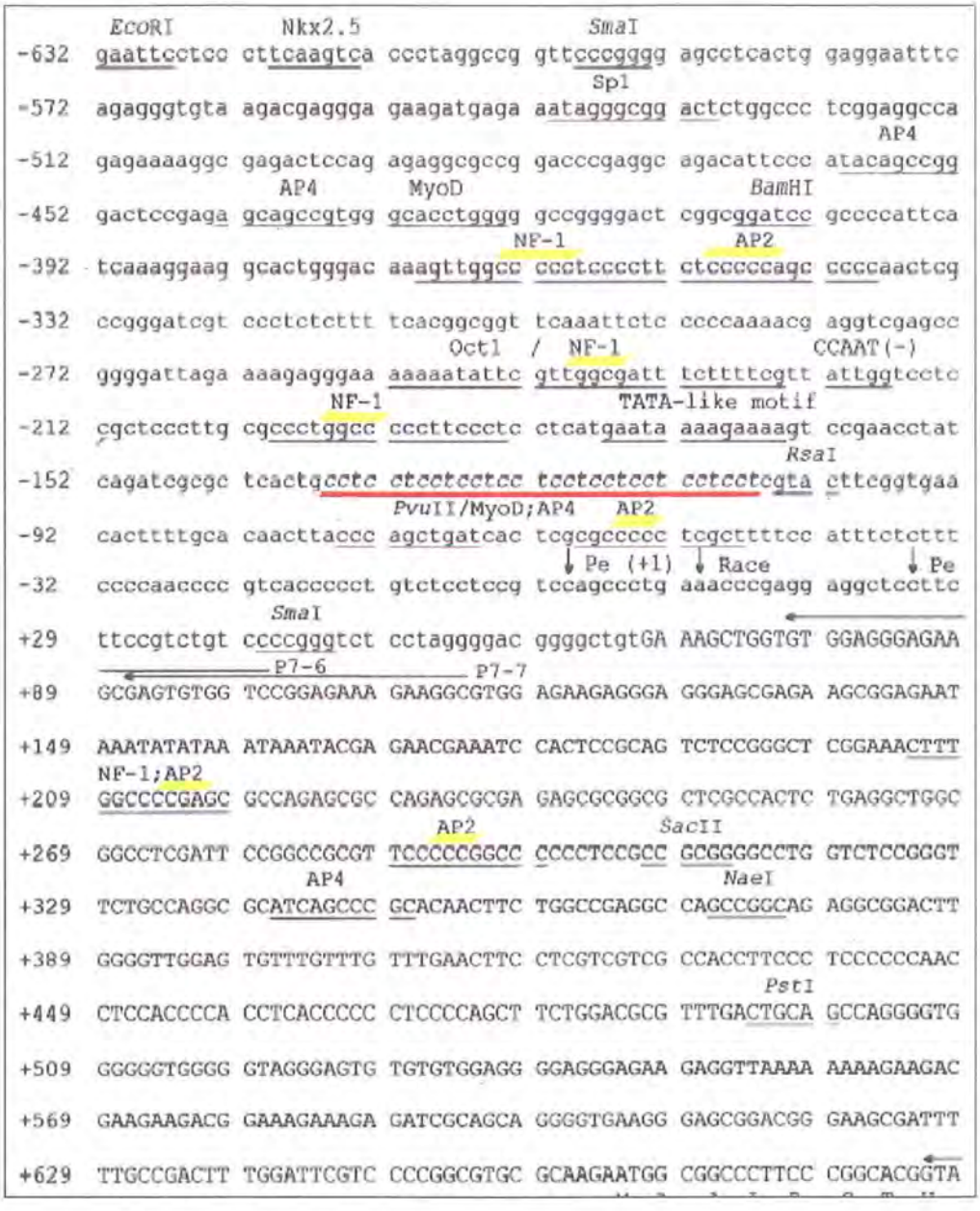


Figure 5.2: Nucleotide sequence of the partial 5' flanking region of the *PAX7* gene (Syagailo et al., 2002). AP2 and NF1 specific DNA binding sequences are indicated.

Analysis of transcription rates *in vitro* may not be accurate as they are dependant on the presence of co-activator transcription factors in the nucleus at the time of assay. Alternatively, while the increased length of the promoter in *PAX7* may be associated with increased rate of *PAX7* transcription, an increased level of PAX7 may be associated with

a decrease in activation of satellite cells. Recent literature suggests that *PAX* genes are associated with maintenance of a stem-cell like state in precursor cells (Lang, Lu, Huang, Engleka, Zhang, Chu, Lipner, Skoultchi, Millar & Epstain, 2005). This may indicate that higher levels of PAX7 prevent satellite cell activation and differentiation and prolong the precursor cell state.

As could be predicted, our results indicate that the intron polymorphism is not associated with recovery of force following damage by eccentric exercise in the Australian and Japanese population groups. Specifically, there is a trend towards high responders being associated with alleles 3 and 4 in the Australian and Japanese groups respectively. A similar trend was found with low responders being associated with allele 4 in the Japanese group (no low responder Australians were found); these results indicate no association between allelic forms and regenerative response. Therefore, the length of the intron polymorphism (whether increased or decreased) has no effect on transcriptional efficiency *in vivo*, resulting in no clear association with regenerative ability.

In order to determine the functional importance of the promoter versus the intron gene regions, we checked whether these gene regions were conserved in other mammalian species. The sequence of the promoter region -57 to -226 bp and the whole intron three sequence were submitted to GenBank and a BLAST analysis was performed, to ascertain the occurrence of similar sequences in other species. As presented in Figure 5.3, the *PAX7* promoter region appears to be highly conserved in mice and zebrafish signifying functional importance of this gene region (70 % conservation in mice and zebrafish). It is

important to note that the promoter region is highly conserved on either side of the CCT repeat, the areas bound by the AP2 and NF1 transcription factors, indicated in Figure 5.2.

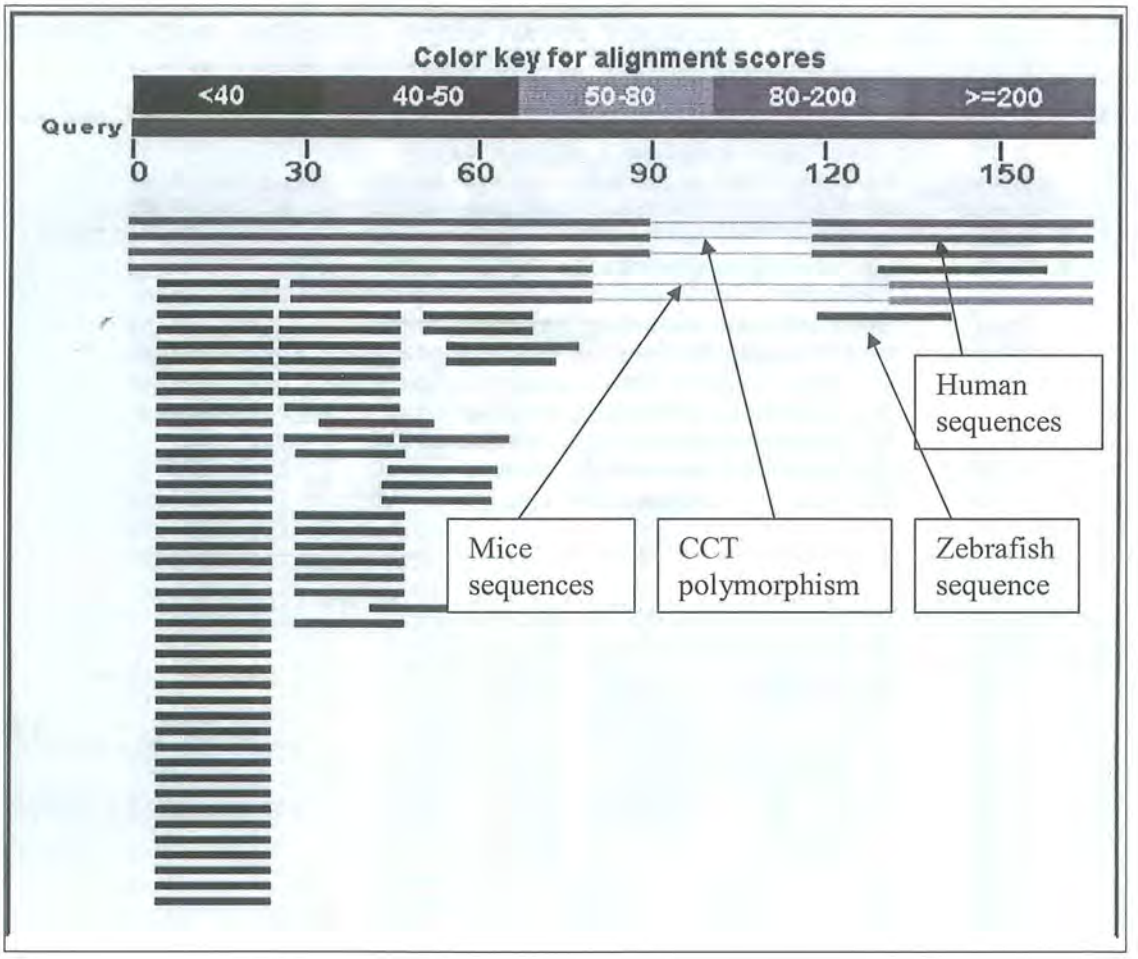


Figure 5.3: Results of BLAST for the promoter region of *PAX7* indicating a high level of conservation.

On the other hand, our results indicate that intron three is not significantly conserved; no similar sequences were observed in other species (Figure 5.4). Clearly then, the promoter polymorphism may have a higher impact on transcriptional activity than the intron polymorphism, by virtue of its location and evolutionary conservation. Our results showing a possible association between regenerative ability and the *PAX7* promoter

polymorphism but not the intron polymorphism. This is consistent with the idea that the promoter region polymorphic locus may be functionally significant whereas the intron polymorphism may not be functionally significant.

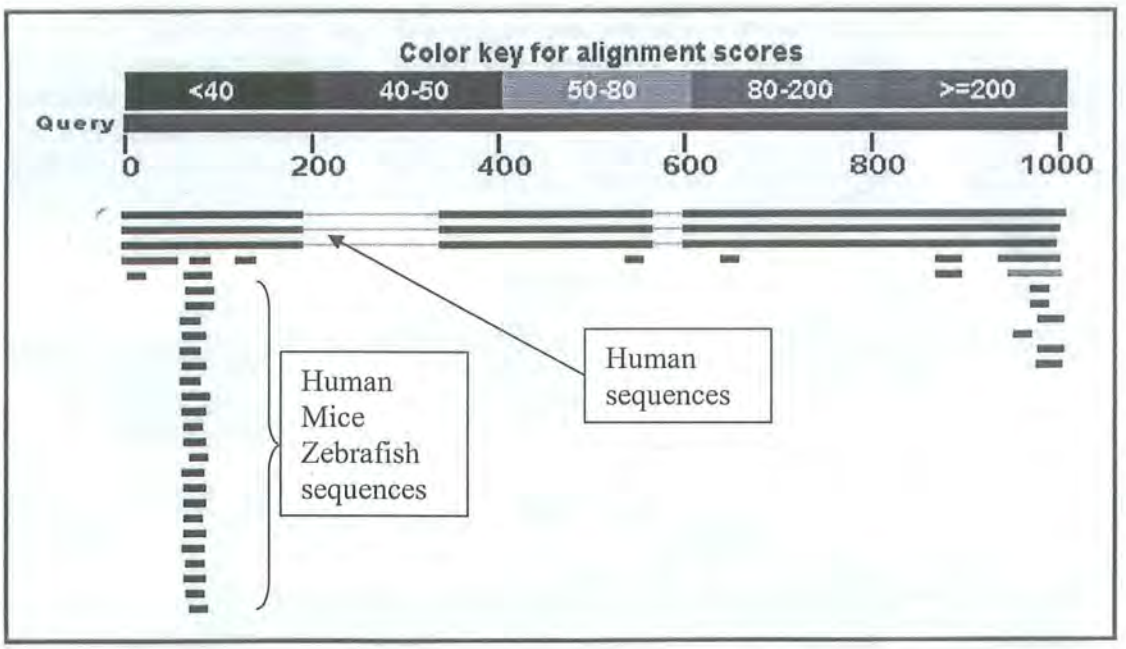


Figure 5.4: Results of BLAST for intron three of *PAX7* which appears not to be conserved.

Given our hypothesis that the *PAX7* promoter allelic variation may contribute to the observed differences in recovery of force following damage by eccentric exercise, it raises the question of whether we can develop specific exercise programs tailored for specific genotypes. Simple genetic tests can be introduced into clinics to analyze genotypes and the sport professionals can develop specific eccentric exercise programs for specific genotypes in order to achieve optimum recovery after muscle damage, resulting in possible performance enhancement. This may add to the new area of sports

science genetics, a field that is growing rapidly as more people search for genetic associations with performance.

The severe limitation in the study was the low subject numbers, making it difficult to accurately validate an association between muscle regenerative ability and allelic variation at the *PAX7* promoter and intron three loci. It was difficult to find volunteers from the Australian and Japanese population groups that were willing to perform the eccentric exercise component of the study, as this is an extremely painful and time consuming exercise.

If we are able to estimate the ability to recover muscle function, this measurement may be useful for assessment of functional recovery after injury associated with muscle pathologies and may eventually be used for disease treatment. Previous experiments have shown that dystrophin-deficient mice are susceptible to eccentric exercise-induced muscle damage (Moens, Baatsen & Marechal, 1993). It is possible that the regenerative changes in people suffering from Duchenne Muscular Dystrophy for example, may be initiated by eccentric contractions (Proske & Morgan, 2001). *PAX7* genotype-specific mild eccentric exercise programs could be developed to effect muscle adaptation process, hoping to achieve better muscle regeneration.

Alternate *PAX7* allelic forms have been observed in both humans and mice. In mice, it is evident that these alternate allelic forms are associated with skeletal muscle regenerative ability after mechanical injury (Kay et al., 1998). Thus it seems possible that similarly, alternate allelic forms of *PAX7* may be associated with skeletal muscle regenerative

ability in humans. In future, knowledge of *PAX7* allele variation may be used as a tool for detection of neuromuscular disease susceptibility.

5.3 Future research

Future studies will include increased sample size to ascertain conclusively whether there is association between *PAX7* alleles and muscle regeneration. Thereafter we will analyze mRNA and protein levels in muscle biopsies from people of different genotypes in random population groups. Further work is also required to perfect the Real Time PCR assay, as this would substantially reduce the cost and time of the genotype analysis and allow it to be adopted universally for other genotyping studies involving *PAX7* or other similar VNTR analyses.

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